DESCRIPTION

KIT FOR IMMOBILIZING ORGANIC SUBSTANCE, ORGANIC SUBSTANCE-IMMOBILIZED STRUCTURE, AND MANUFACTURING METHODS THEREFOR

TECHNICAL FIELD

The present invention relates to an organic substance-immobilized substrate prepared by immobilizing an organic substance on a substrate's surface at least part of which has an aluminum oxide layer and to a method of manufacturing the same, and further relates to a peptide having an affinity to a layer containing aluminum oxide and used for immobilization of the organic substance and to a DNA that encodes the peptide having the affinity. More particularly, the present invention relates to a biological substance-immobilized substrate which is applicable to a target substance-detecting element, a target substance-converting element, a target substance-separating element, and a protein structure-optimizing element by the utilization of a biological substance immobilized on the substrate, and to a manufacturing method therefor.

BACKGROUND ART

Many studies and developments have been

conducted for a wide range of applications of so-called biosensors and bioreactors that utilize molecular recognition ability and substance-converting ability of biological substances such as enzymes and antibodies as well as nucleic acid molecules (e.g., DNA and RNA) including genes.

For the biosensors, there are growing demands on further technical developments for applications on various detection targets in conjunction with growing interests in health as well as matters of environmental pollutants and public safety thereof. Recently, furthermore, the bioreactors have attracted much attention as eco-friendly clean processing technologies. Therefore, for example, there are increasing demands on further technical developments such as those in processes of producing products utilizing various bioprocesses.

For the biosensors, specifically, detectors for detecting objective ones by utilizing the selective molecular recognition of respective biological substance molecules have been developed extensively. For example, detectors developed on the basis of various kinds of detection procedures include a DNA sensor chip that utilizes a base-sequence-dependent complimentary hydrogen bonding between deoxyribonucleic acid (hereinafter, referred to as DNA) sequences (i.e., a hybridization reaction

between the complimentary strands), an antibody sensor that detects a disease marker or the like to be eluted in blood, by utilizing a molecular recognition ability, originated from a specific binding ability between a protein molecule and a low-molecular substance or between protein molecules such as an antigen-antibody reaction, and an enzyme sensor for detecting the level of a substrate substance by utilizing an oxidation-reduction enzyme or a hydrolytic enzyme, as typified by a glucose sensor for a diabetic patient.

Currently, the biosensor that makes use of any of these biological substances, generally employs a system of using, in the form of a biological substance-immobilized substrate, a biological substance to be used, for example a nucleic acid molecule such as DNA, or proteins of antibodies, enzymes, etc., which is immobilized on the surface of a substrate such as a flat plate, a sphere or a materials, or the like.

In addition, one of the performance qualities required for the biosensors being developed nowadays is "high sensitivity and downsizing", which is typified by μ-TAS. For attaining an object of "high sensitivity and downsizing", an important technical issue is how to effectively utilize a minute space of a reaction field or detection field and how to

increase the sensitivity of the biosensor.

For instance, in the detection field where the biological substance is immobilized on the substrate, in addition to the specific binding to a target substance to be detected, there is a possibility of causing much non-specific adsorption of biological substances except the substance to be detected or a possibility of causing a non-specific binding of the substance to be detected itself on the substrate. These non-specific adsorbing phenomena will become one of the factors that decrease a Signal/Noise ratio of the biosensor. In particular, the total amount of the specific binding of the target substance to be detected falls off as the detection field decreases. Therefore, the biosensor tends to be influenced by noises due to the non-specific adsorption, resulting in difficulty in high sensitive measurement. Also, in terms of an effective utilization of a sample in minute amounts, it is difficult to carry out measurement at a sufficiently high accuracy when the non-specific adsorption of the target substance to be detected is caused in large quantities. Hereafter, therefore, an important technical problem which remains to be improved is to reduce or prevent the non-specific adsorption phenomenon.

On the other hand, for the bioreactors, there have developed procedures for producing food

additives such as amino acids, candidate substances for medicines and antibiotics by enzymatic reactions that mainly employ the position-selective catalytic functions of enzymes as one type of proteins instead of procedures that utilize microorganisms having the abilities of producing objective products. Besides, the applications of enzymatic reactions to the productions of chemicals and polymer materials have been also under study. In the development of bioreactors using such enzymatic reactions, because the development of devices suitable for high-mix low-volume production has been also mainstream, for example, with the spread of a technique for screening a candidate substance by means of combinatorial chemistry, there are increasing demands for miniaturizing individual biosensors by means of a device on which an enzyme to be used in a reaction just as in the case with the biosensor described above is immobilized (i.e., for high-mix low-volume production).

In addition, materials, which can be employed for substrates, flat plates, spheres and porous materials, or the like, for biological substance-immobilized substrates to be used in the biosensors and bioreactors, generally include organic polymers, glass, ceramics, metal flat plates, and other materials known in the art, depending on the types

and applications of the biological substances.

As a method of immobilizing a biological substance such as protein, on the surface of substrate, for example, there is an immobilizing procedure using physical adsorption, which includes the steps of forming a coating layer of a protein solution on the surface of a substrate by using means for dipping the substrate into the protein solution or applying the protein solution thereon, and then removing a solvent from the coating layer and drying it to allow the protein to be immobilized on the surface of the substrate as a result of physical adsorption. Alternatively, there is another procedure that includes next two steps, the first stepis chemically modifying the surfaces of a substrate or the protein molecules to provide the high activity functional groups, and second step is immobilization of the protein molecules on the surface of the substrate through the chemical bonding by forming of chemical bonding between the introduced the high activity functional groups and other functional groups. These procedures have been hitherto known as those for immobilizing biological substances on the surfaces of substrate. As an example of the immobilizing method using physical adsorption, JP 06-003317 A discloses a method of manufacturing an enzymatic electrode by the

application of a method including the steps of forming a charge-transporting organic complex layer on the surface of a conductive substrate and then applying a protein solution on the charge-transporting organic complex layer, followed by drying the protein layer to allow an enzyme protein to be physically adsorbed and immobilized on the surface of the substrate through the charge-transporting organic complex layer.

As an example of the immobilizing method using the chemical bonding, Sensor and Actuators B15-16 p127 (1993) discloses a method including the steps of subjecting a platinum-deposited surface of a silicon substrate to treatment with an amine-based silane coupling agent and then coupling between an amino group derived from the amino-silane coupling and a peptide chain by means of a cross-linking agent such as glutaric aldehyde to carry out immobilization. In addition, for making a detector such as a biosensor composed of antibodies immobilized on a glass substrate, a method is applied, in which reactive functional groups are introduced to the surface of the glass substrate by means of treatment with a silane coupling agent and a peptide chain is immobilized through a chemical bonding using a cross-lining agent as described above.

However, in the immobilizing method based on

physical adsorption or the chemically-immobilizing method based on chemical cross-linking, the portion of a protein, which is used for adsorption or binding to the substrate can be selected at random. Therefore, when a portion, which directly or indirectly relates to the binding ability required for the protein, the enzymatic activity of the protein, or the like, also becomes one relating to the binding to the surface of a substrate, there is a fear that a desired function of the protein will deteriorate remarkably if the protein binds to the substrate.

Therefore, it becomes important to develop means for previously determining an immobilizing portion of a molecule to be immobilized, which will be used for binding to the surface of the substrate, for example, a technology capable of previously controlling the orientation of a biological substance to be immobilized on the surface of the substrate.

Furthermore, for attaining "high sensitivity and downsizing", the biological substance should be integrated very densely in a very small area on the surface of the substrate and then immobilized thereon.

As an example of a method of integrating the biological substance very densely and immobilizing the same, there is a method well known in the art, where a substrate having a large specific surface

area, for example a porous material having a regular nanoporous structure, is adopted as a substrate, and a biological substance is then immobilized on the surface having a porous structure with a large specific surface area. As a conventional method for forming the porous structure having regularity with a scale in the order of nanometers, which can be used for the above purpose, a polymer membrane filter, porous glass, anodized aluminum oxide film, and so on are well known in the art. For the anodized aluminum oxide film, in particular, the pore size thereof can be regulated by means of a voltage applied at the time of oxidation to make a film having a given pore size in the order of nanometers.

Making the porous material into the substrate enables a reaction field on which a biological substance is immobilized in an amount enough for high-sensitivity detection even in a very small area.

Conventional examples of the method using the porous substrate described above as a substrate, particularly the method by which a biological substance such as a protein is immobilized on an anodized aluminum oxide film, include the following procedures:

As an example of a procedure for covalently binding a protein using a cross-linking agent after surface treatment with an amino-silane coupling agent,

USP 6,225,131 discloses a method including the steps of providing a commercially-available aluminum oxide film as a substrate, subjecting the surface thereof to treatment with 3-aminopropyltriethoxysilane (APS), and covalently binding anti-human chorionic gonadotropin mouse monoclonal antibodies using glutaric aldehyde as a cross-linking agent to immobilize them on the surface of the substrate.

Furthermore, as an example of a procedure using intermolecular binding between an organic substance and a peptide, US 2002/0106702 A1 discloses a method by which an organic thin film for binding a protein is arranged on an aluminum oxide film to immobilize a protein fused with a peptide chain having affinity to an organic substrate that constitutes an organic thin film described above.

The above substrate having a large specific surface area, such as a porous material having a regular nanoporous structure, is adopted as a substrate to allow a larger amount of the biological substance to be immobilized on the surface of the substrate. However, when the biological substance immobilized on the substrate does not take an orientation suitable for the binding to a target substance to be detected, the detection sensitivity corresponding to the amount of the biological substance immobilized may not be attained. Also,

when a biological substance does not have an orientation suitable for the substrate substance on which the biological substance acts, the reactivity corresponding to an amount of the biological substance to be immobilized is not attained in some cases. That is, unless a biological substance to be immobilized on a substrate is immobilized after controlling the orientation suitable for the use thereof, the biological substance will be insufficient to exert its advantage accompanying immobilization of a larger amount of the biological substance on the surface of the substrate through the use of a substrate having a large specific surface area.

In other words, unless a biological substance to be immobilized on a substrate is immobilized after controlling the orientation suitable for the use thereof, it becomes necessary to further increase the amount of the biological substance to be immobilized on the substrate to obtain the desired detection sensitivity or reactivity. Thus, there is a possibility that an excess amount of the biological substance per unit area of the substrate should be immobilized or the area of the substrate on which the biological substance is immobilized should be excessively extended. When the area of the substrate on which the biological substance is immobilized is

extended excessively, it may become a large obstacle to the downsizing of a device itself.

Furthermore, in the case of ingredients in the biological substance, which will cost high upon their preparation, there is a possibility of increasing the total cost of the device when they will be used in large amounts. Furthermore, a procedure of forming an additional adhesion layer for binding an organic substance to a substrate (i.e., a layer formed between the substrate and the organic substance and having a configuration different from that of the organic substance to be immobilized) may involve an increase in the number of steps required and become a large obstacle to a decrease in device cost.

In addition, a high technical level is also required for completely forming the adhesion layer on the inner-wall surface of the porous portion of the nanoporous structure. If the formation of the adhesion layer in the inside of the pore is insufficient, an effect obtained by increasing the specific surface area by means of the porous structure may be insufficiently exerted.

In view of the present situation, such a problem cannot be coped with any publicly known technology of immobilizing ingredients in the biological substance by means of chemical bonding between the ingredients and the substrate with

physical adsorption through the adhesion layer or non-specific modification using a cross-linking agent.

Therefore, it has been desired to provide a structure composed of a substrate and an organic substance immobilized on the surface of the substrate such that the molecular orientation of the organic substance is regulated so as to exert its desired functions, and a concise immobilizing procedure that allows the organic substance to be immobilized on the surface of the substrate.

DISCLOSURE OF THE INVENTION

An object of the present invention is to provide an organic substance-immobilized structure that adopts novel immobilizing means that enables stable immobilization of an organic substance used for a biosensor or bioreactor on the surface of a substrate with orientation suitable for exerting physiological functions of the organic substance, especially, biological substance when the organic substance, particularly the biological substance, is immobilized on the surface of the substrate, and a manufacturing method utilizing the novel immobilizing means. In addition, another object of the present invention is to provide a peptide having an affinity to an aluminum oxide-containing layer which is available as the novel immobilizing means and a DNA

that encodes the peptide, and an expression vector for providing a fused product of the organic substance and a binding domain containing the peptide having the affinity to aluminum oxide. In addition, still another object of the present invention is to provide a fused product of an organic substance and a binding domain.

For attaining the above objects, the inventors of the present invention have made studies on novel immobilizing means which is available when an organic substance, especially biological substance is immobilized on the surface of a substrate and found that the organic substance may be stably immobilized on the surface of a substrate with an orientation suitable for exerting physiological functions of the organic substance, particularly the biological substance, when a portion used for immobilization is arranged separately from a main portion of the organic substance, and a procedure utilizing a physical interaction specific to the portion to be utilized in immobilization instead of a procedure utilizing a chemical reagent, is chosen for binding to the surface of the substrate in the portion utilized in immobilization in order to allow an immobilized organic substance, particularly a biological substance, to exert sufficiently original physiological functions.

In the procedure that utilizes the physical interaction specific to the portion utilized for immobilization, a peptide having an affinity to aluminum oxide can be selected when a substrate containing aluminum oxide is used as a material of the surface to be immobilized. It was confirmed that an organic substance, particularly a biological substance, may be immobilized on the substrate having the surface made of an aluminum-oxide-containing material through a physical interaction between the aluminum oxide on the surface and the binding domain having an affinity to the aluminum oxide with high reproducibility and stability while keeping its orientation suitable for exerting physiological functions thereof when a binding domain containing the peptide having the affinity to aluminum oxide is in the form of a fused product coupled with a functional domain made of the organic substance, particularly the biological substance.

The present invention has been completed on the basis of those findings.

That is, according to the present invention, an organic substance-immobilized structure includes:

a substrate having an organic substance immobilized on a surface thereof and the surface at least part of which contains aluminum oxide; and

a binding domain for immobilizing the organic

substance on the substrate, having an ability to bind to the aluminum oxide and being coupled with the organic substance, wherein:

the binding domain contains at least a peptide composed of one or more amino acids; and

the organic substance is immobilized on the surface of the substrate through the binding domain by means of specific binding of the peptide to the aluminum oxide.

In the organic substance-immobilized structure according to the present invention, a capture molecule for capturing a target substance can be used as the organic substance. Alternatively, as the organic substance, a molecule having a function to convert the substance can be used.

In addition, the present invention also provides a method of manufacturing the organic substance-immobilized structure. That is, the method of manufacturing the organic substance-immobilized structure according to the present invention is a method of manufacturing a structure having an organic substance immobilized on a substrate, including the steps of:

preparing an organic substance - binding domain fused product composed of the substrate having a surface at least part of which contains aluminum oxide and a binding domain, having an ability to bind

to the aluminum oxide and coupled with the organic substance; and

immobilizing the organic substance on the surface of the substrate by bringing the fused product into contact with the surface of the substrate to cause a peptide having an ability to bind to the aluminum oxide to specifically bind to the aluminum oxide.

In this case, the organic substance may be a biological substance containing a protein. The method may further include the step of obtaining the organic substance - binding domain fused product by inducing expression of a fused product-type protein formed by coupling a peptide portion included in the binding domain with the protein included in the biological substance on the basis of a coupling gene having a sequence of bases coupled with each other so as to encode a combination of an amino acid sequence of the protein and an amino acid sequence included in the binding domain which are coupled.

On the other hand, a peptide having an ability to bind to aluminum oxide, which is a feature of the organic substance-immobilized structure of the present invention, is a peptide, which has any one of at least one amino acid sequence selected from the group consisting of amino acid sequences of SEQ ID NOS: 1 to 32, an altered amino acid sequence obtained

such that the amino acid sequence is subjected to a deletion, substitution, or addition of one or more amino acids, and a complex amino acid sequence containing two or more of these amino acid sequences, and a repetitive sequence of the amino acid sequences,

the amino acid sequence including a peptide having an affinity to aluminum oxide.

Val-Tyr-Ala-Asn-Gln-Thr-Pro-Pro-Ser-Lys-Ala-Arg (SEQ ID NO: 1)

Gln-Ser-Ser-Ile-Thr-Thr-Arg-Asn-Pro-Phe-Met-Thr (SEQ ID NO: 2)

Phe-Met-Asn-His-His-Pro-Asn-Ser-Gln-Gln-Tyr-His (SEQ ID NO: 3)

Gln-Tyr-Thr-Ser-Ser-Gly-Ile-Ile-Thr-Ser-Ser-Ala (SEQ ID NO: 4)

His-His-His-Pro-Glu-Asn-Leu-Asp-Ser-Thr-Phe-Gln (SEQ ID NO: 5)

Gln-Pro-His-Met-His-Arg-Ser-Ser-His-Gln-Asp-Gly (SEQ ID NO: 6)

Asn-Thr-Thr-Met-Gly-Pro-Met-Ser-Pro-His-Ser-Gln (SEQ ID NO: 7)

Ala-Ala-His-Phe-Glu-Pro-Gln-Thr-Met-Pro-Met-Ile (SEQ ID NO: 8)

Asp-His-Gln-Leu-His-Arg-Pro-Pro-His-Met-Met-Arg (SEQ ID NO: 9)

Val-Ser-Arg-His-Gln-Ser-Trp-His-Pro-His-Asp-Leu (SEQ ID NO: 10)

Met-Met-Gln-Arg-Asp-His-His-Gln-His-Asn-Ala-Gln (SEQ ID NO: 11)

Val-Thr-Leu-His-Thr-Val-Asp-His-Ala-Pro-Gln-Asp (SEQ ID NO: 12)

Ser-Val-Ser-Val-Gly-Met-Lys-Pro-Ser-Pro-Arg-Pro (SEQ ID NO: 13)

His-Leu-Gln-Ser-Met-Lys-Pro-Arg-Thr-His-Val-Leu (SEQ ID NO: 14)

Ile-Pro-Asn-Ala-Glu-Thr-Leu-Arg-Gln-Pro-Ala-Arg (SEQ ID NO: 15)

Val-Gly-Val-Ile-Ser-Ser-Trp-His-Pro-His-Asp-Leu (SEQ ID NO: 16)

Thr-Val-Pro-Ile-Tyr-Asn-Thr-Gly-Ile-Leu-Pro-Thr (SEQ ID NO: 17)

Tyr-Thr-Met-His-His-Gly-Ser-Thr-Phe-Met-Arg-Arg (SEQ ID NO: 18)

Ser-Met-Met-His-Val-Asn-Ile-Arg-Leu-Gly-Ile-Leu (SEQ ID NO: 19)

Ala-Pro-Met-His-His-Met-Lys-Ser-Leu-Tyr-Arg-Ala (SEQ ID NO: 20)

Met-Met-Gln-Arg-Asp-His-His-Gln-His-Met-Arg-Arg (SEQ ID NO: 21)

Met-Lys-Thr-His-His-Gly-Asn-Asn-Ala-Val-Phe-Leu (SEQ ID NO: 22)

Leu-Glu-Pro-Leu-Pro-His-Thr-Pro-Arg-Met-Tyr-Ala (SEQ ID NO: 23)

Gln-Leu-Tyr-Glu-Pro-Asp-Ser-Gly-Pro-Trp-Ala-Pro (SEQ

ID NO: 24)

Trp-Met-Thr-Lys-Met-Pro-Thr-Thr-His-Thr-Arg-Tyr (SEQ ID NO: 25)

His-His-Pro-Met-Tyr-Ser-Met-Thr-Arg-Ala-Leu-Pro (SEQ ID NO: 26)

Gly-Ser-Ala-His-Ser-Arg-Asn-Asp-Ala-Ala-Pro-Val (SEQ ID NO: 27)

His-Ser-Pro-Leu-Met-Gln-Tyr-His-Met-Ser-Gly-Thr (SEQ ID NO: 28)

Thr-Ala-His-Met-Thr-Met-Pro-Ser-Arg-Phe-Leu-Pro (SEQ ID NO: 29)

Ala-Cys-Pro-Pro-Thr-Gln-Ser-Arg-Tyr-Cys (SEQ ID NO: 30)

Ala-Cys-Asn-Gly-Met-Leu-Ala-Phe-Gln-Cys (SEQ ID NO: 31)

Ala-Cys-Thr-Pro-Lys-Pro-Gly-Lys-His-Cys (SEQ ID NO: 32)

In addition, the present invention provides a DNA molecule to be used in making a peptide chain having an ability to bind to the aluminum oxide as a part of a fused product-type protein in the method of manufacturing the organic substance-immobilized structure.

In other words, the DNA molecule that encodes the peptide chain having the ability to bind to aluminum oxide according to the present invention is a DNA molecule, which encodes a peptide chain,

the peptide chain having any one of at least one amino acid sequence selected from the group consisting of amino acid sequences of SEQ ID NOS: 1 to 32, an altered amino acid sequence obtained such that the amino acid sequence is subjected to a deletion, substitution, or addition of one or more amino acids, and a complex amino acid sequence containing two or more of the amino acid sequences, and a repetitive sequence of any of the amino acid sequences,

the amino acid sequence having an affinity to aluminum oxide.

In addition, the present invention also provides an expression vector to be used for expression of a fused product-type protein that includes a peptide chain having an ability to bind to the aluminum oxide in the method of manufacturing the organic substance-immobilized structure. That is, the expression vector according to the present invention includes a coupling gene, wherein the coupling gene can induce expression of a fused product-type protein formed by coupling a peptide portion included in a binding domain with a protein included in an organic substance in a host cell with respect to an organic substance - binding domain fused product composed of:

the organic substance containing a protein in

at least part thereof; and

the binding domain coupled with the organic substance, containing at least a peptide having an affinity to aluminum oxide and made of one or more amino acids and having an ability to bind to the aluminum oxide, with the peptide having the affinity to the aluminum oxide containing any one of at least one amino acid sequence selected from the group consisting of amino acid sequences of SEQ ID NOS: 1 to 32, an altered amino acid sequence obtained such that the amino acid sequence is subjected to a deletion, substitution, and addition of one or several amino acids, or a complex amino acid sequence containing two or more of the amino acid sequences, and a repetitive sequence of the amino acid sequences,

on the basis of a coupling gene having a sequence of bases being coupled with each other to encode a combination of an amino acid sequence of the protein and an amino acid sequence included in the binding domain, which constitute the organic substance - binding domain fused product.

Therefore, the expression vector according to the present invention can be provided as an expression vector made by inserting DNA by which at least amino acid sequence of a fused product-type protein among peptide chains that constitute the organic substance - binding domain fused product into

various vectors, for example plasmids, phagemids, and cosmids, to be used in molecular biological procedures such as transformation of host cells by transferring genes into the host cells and expression of proteins.

Furthermore, the present invention also provides a kit for manufacturing an organic substance-immobilized structure exclusively used for the manufacture of the organic substance-immobilized structure according to the present invention. That is, the kit for manufacturing the organic substance-immobilized structure according to the present invention is a kit for immobilizing an organic substance on a substrate, including:

a substrate having a surface at least part of which contains aluminum oxide; and

a binding domain for immobilizing the organic substance on the substrate, having an ability to bind to the aluminum oxide and being coupled with the organic substance, wherein:

the binding domain contains at least a peptide composed of one or more amino acids; and

the organic substance is immobilized on the substrate by means of specific binding of the peptide to the aluminum oxide when the substrate and the binding domain are brought into contact with each other.

In addition, an organic substance - binding domain fused product contains an organic substance and a binding domain having an ability to bind to aluminum oxide, wherein the binding domain contains at least a peptide composed of one or more amino acids, and the peptide contains any one of at least one amino acid sequence selected from the group consisting of amino acid sequences of SEQ ID NOS: 1 to 32, an altered amino acid sequence obtained such that the amino acid sequence is subjected to a deletion, substitution, or addition of one or several amino acids, and a complex amino acid sequence containing two or more of the amino acid sequences, and a repetitive sequence of the amino acid sequences.

Furthermore, in the organic substance-immobilized structure according to the present invention, for example a substrate on which an organic substance is immobilized, an aluminum oxide layer is formed to form a surface of the substrate on which the organic substance is to be immobilized, while the organic substance to be immobilized is provided itself as a functional domain and is constructed such that a binding domain formed of a peptide constructed of one or more amino acids having an ability to bind to the aluminum oxide layer is coupled with the functional domain. Thus, the organic substance provided as the functional domain

is allowed to be selectively immobilized on the surface of the substrate through the ability of the binding domain coupled to bind to aluminum oxide without directly contacting the surface of the substrate. The organic substance is immobilized on the surface of the substrate through the binding domain formed independently, so that the functions inherent in the organic substance will not be influenced by immobilization and also any chemical reagent is not used in the immobilization. Therefore, the organic substance is not subjected to a chemical reaction that affects the desired function of the organic substance.

In the organic substance-immobilized structure according to the present invention, an amino acid sequence of a peptide included in the binding domain is appropriately selected from peptides having an affinity to the aluminum oxide of the present invention. Thus, a fused product can be provided as one having the functions of the organic substance, particularly the biological substance, as an immobilization target, which are comparable with the inherent functional levels at all, allowing the organic substance to be used for various immobilization target organic substrates. Besides, the functions of the organic substance to be immobilized are selected and used, so the organic

substance-immobilized structure of the present invention can be applied to a more excellent target substance-capturing element, target substance-converting element, target substance-separating element, and protein structure-optimizing element.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a cross sectional diagram schematically showing a configuration of a structure of an example of an organic substance-immobilized structure according to the present invention;

Fig. 2 is a cross sectional diagram schematically showing the configuration of the structure of an example of the organic substance-immobilized structure according to the present invention;

Fig. 3 is a cross sectional diagram schematically showing the configuration of the structure of an example of the organic substance-immobilized structure according to the present invention;

Fig. 4 is a diagram showing primer configurations used for synthesis of a long-chain DNA by PCR used in Example 4 and synthetic steps (Steps 1 to 4); and

Fig. 5 is a diagram showing the primer configurations used for synthesis of a long-chain DNA

by PCR used in Example 5 and synthetic steps (Steps 1 to 5).

BEST MODE FOR CARRYING OUT THE INVENTION

The organic substance-immobilized structure of the present invention is a structure where an organic substance is immobilized on the surface of the substrate, characterized by including: a substrate having a surface at least part of which contains aluminum oxide; and an organic substance being immobilized on the surface of the substrate through a binding domain containing a peptide composed of at least one or more amino acids. In particular, the peptide included in the binding domain is characterized by containing an amino acid sequence having an ability to bind to the aluminum oxide.

On the other hand, the organic substance may include a biological substance and the biological substance may include a protein or a part thereof. At this time, in a coupling portion between the biological substance and the binding domain, a linker composed of one or more amino acids may be included between a peptide containing an amino acid sequence having an ability to bind to the aluminum oxide in the binding domain and the biological substance.

The peptide, which contains the amino acid sequence having the ability to bind to the aluminum

oxide in the binding domain, is a peptide containing the whole of at least one amino acid sequence selected from the group consisting of SEQ ID NOS: 1 to 32 described below, or an amino acid sequence having a part thereof, or a peptide having a repetitive structure of any of these amino acid sequences, or a peptide containing a complex composed of these amino acid sequences, and alternatively the peptide may contain one or more sequences of the amino acid sequences of SEQ ID NOS: 30 to 32 and may form a cyclic structure with an intramolecular disulfide binding in the sequence.

Val-Tyr-Ala-Asn-Gln-Thr-Pro-Pro-Ser-Lys-Ala-Arg (SEQ ID NO: 1)

Gln-Ser-Ser-Ile-Thr-Thr-Arg-Asn-Pro-Phe-Met-Thr (SEQ ID NO: 2)

Phe-Met-Asn-His-His-Pro-Asn-Ser-Gln-Gln-Tyr-His (SEQ ID NO: 3)

Gln-Tyr-Thr-Ser-Ser-Gly-Ile-Ile-Thr-Ser-Ser-Ala (SEQ ID NO: 4)

His-His-His-Pro-Glu-Asn-Leu-Asp-Ser-Thr-Phe-Gln (SEQ ID NO: 5)

Gln-Pro-His-Met-His-Arg-Ser-Ser-His-Gln-Asp-Gly (SEQ ID NO: 6)

Asn-Thr-Thr-Met-Gly-Pro-Met-Ser-Pro-His-Ser-Gln (SEQ ID NO: 7)

Ala-Ala-His-Phe-Glu-Pro-Gln-Thr-Met-Pro-Met-Ile (SEQ

ID NO: 8)

Asp-His-Gln-Leu-His-Arg-Pro-Pro-His-Met-Met-Arg (SEQ ID NO: 9)

Val-Ser-Arg-His-Gln-Ser-Trp-His-Pro-His-Asp-Leu (SEQ ID NO: 10)

Met-Met-Gln-Arg-Asp-His-His-Gln-His-Asn-Ala-Gln (SEQ ID NO: 11)

Val-Thr-Leu-His-Thr-Val-Asp-His-Ala-Pro-Gln-Asp (SEQ ID NO: 12)

Ser-Val-Ser-Val-Gly-Met-Lys-Pro-Ser-Pro-Arg-Pro (SEQ ID NO: 13)

His-Leu-Gln-Ser-Met-Lys-Pro-Arg-Thr-His-Val-Leu (SEQ ID NO: 14)

Ile-Pro-Asn-Ala-Glu-Thr-Leu-Arg-Gln-Pro-Ala-Arg (SEQ ID NO: 15)

Val-Gly-Val-Ile-Ser-Ser-Trp-His-Pro-His-Asp-Leu (SEQ ID NO: 16)

Thr-Val-Pro-Ile-Tyr-Asn-Thr-Gly-Ile-Leu-Pro-Thr (SEQ ID NO: 17)

Tyr-Thr-Met-His-His-Gly-Ser-Thr-Phe-Met-Arg-Arg (SEQ ID NO: 18)

Ser-Met-Met-His-Val-Asn-Ile-Arg-Leu-Gly-Ile-Leu (SEQ ID NO: 19)

Ala-Pro-Met-His-His-Met-Lys-Ser-Leu-Tyr-Arg-Ala (SEQ ID NO: 20)

Met-Met-Gln-Arg-Asp-His-His-Gln-His-Met-Arg-Arg (SEQ ID NO: 21)

Met-Lys-Thr-His-His-Gly-Asn-Asn-Ala-Val-Phe-Leu (SEQ ID NO: 22)

Leu-Glu-Pro-Leu-Pro-His-Thr-Pro-Arg-Met-Tyr-Ala (SEQ ID NO: 23)

Gln-Leu-Tyr-Glu-Pro-Asp-Ser-Gly-Pro-Trp-Ala-Pro (SEQ ID NO: 24)

Trp-Met-Thr-Lys-Met-Pro-Thr-Thr-His-Thr-Arg-Tyr (SEQ ID NO: 25)

His-His-Pro-Met-Tyr-Ser-Met-Thr-Arg-Ala-Leu-Pro (SEQ ID NO: 26)

Gly-Ser-Ala-His-Ser-Arg-Asn-Asp-Ala-Ala-Pro-Val (SEQ ID NO: 27)

His-Ser-Pro-Leu-Met-Gln-Tyr-His-Met-Ser-Gly-Thr (SEQ ID NO: 28)

Thr-Ala-His-Met-Thr-Met-Pro-Ser-Arg-Phe-Leu-Pro (SEQ ID NO: 29)

Ala-Cys-Pro-Pro-Thr-Gln-Ser-Arg-Tyr-Cys (SEQ ID NO: 30)

Ala-Cys-Asn-Gly-Met-Leu-Ala-Phe-Gln-Cys (SEQ ID NO: 31)

Ala-Cys-Thr-Pro-Lys-Pro-Gly-Lys-His-Cys (SEQ ID NO: 32)

That is, for the organic substance-immobilized structure of the present invention, there is used a procedure in which, when an organic substance is immobilized on the surface of a substrate where at least part of the surface thereof contains aluminum

oxide, the binding domain containing the peptide having an amino acid sequence representing an ability to bind to the aluminum oxide is designed to provide an organic substance - binding domain fused product formed by previously binding the binding domain to a desired organic substance to specifically immobilize the organic substance - binding domain fused product to the surface of the substrate containing aluminum oxide through the binding domain portion having an ability to bind to the aluminum oxide.

Therefore, in the organic substance-binding domain fused product formed by previously binding the binding domain, when the organic substance is a biological substance, a chemical reaction that utilizes a reagent that affects the functions (e.g., molecular recognition and catalytic ability) is not used at the time of immobilization of the biological substance-binding domain fused product to the surface of the substrate containing aluminum oxide. Thus, the biological substance to be immobilized will be kept in a state of being able to exert the functions thereof sufficiently.

In addition, depending on an aluminum oxide substrate used for the surface of the substrate, an amino acid sequence having a desired binding ability can be selected in advance through screening. Besides, depending on the objective biological

substance, a design for optimizing the binding configuration of the binding domain to be previously bound to the biological substance and the amino acid sequence representing an ability to bind to aluminum oxide in the binding domain can be worked out. Therefore, the organic substance-immobilized structure according to the present invention can be applied extensively to both the aluminum oxide and the organic substance, particularly the biological substance.

In addition, the manufacturing method of an organic substance-immobilized structure in accordance with the present invention is a method of manufacturing an organic substance-immobilized structure characterized by including:

a substrate having a surface at least part of which contains aluminum oxide; and

an organic substance immobilized on the surface of the substrate through a binding domain containing at least one or more amino acids, the method being characterized by including the steps of:

(1) making an organic substance-binding domain fused product constructed by coupling the binding domain with the organic product; and

(2) immobilizing the organic substance on the surface of the substrate through the binding domain by bringing the organic substance - binding domain

fused product into contact with the surface of the substrate to allow at least part of the binding domain in the organic substance - binding domain fused product to bind to the surface of the substrate.

Furthermore, it is preferable that the organic substance be a biological substance containing a protein and the above step (1) of making the organic substance - binding domain fused product include a step of expressing a fused product-type protein constructed by coupling a peptide portion included in the binding domain with a protein included in the biological substance on the basis of a coupling gene having a base sequence constructed by coupling a base sequence encoding the amino acid sequence of a protein included in the biological substance with a base sequence encoding the amino acid sequence of a peptide portion included in the binding domain to encode a combination of two amino acid sequences described above.

Furthermore, a manufacturing method is one characterized in that the above coupling gene has a base sequence in which a base sequence encoding a linker constructed of one or more amino acids is coupled between the base sequence encoding the amino acid sequence of the protein included in the biological substance and the base sequence encoding the amino acid sequence of the peptide portion

contained in the binding domain so as to encode a combination of two amino acid sequences described above.

Furthermore, depending on the selection of an organic substance used, the organic substance-immobilized structure of the present invention can be a component to be available in a target substance-capturing element, a target substance-detecting element, a substance-converting element, or a target substance-separating element, or available in all of these elements.

Moreover, the present invention utilizes the present invention of a certain amino acid sequence of a peptide in the binding domain used in the configuration of the above organic substance-immobilized structure of the present invention. In other words, the aluminum oxide affinity peptide of the present invention may be an aluminum oxide affinity peptide characterized by including the whole of at least one amino acid sequence or an amino acid sequence containing at least part thereof selected from the group consisting of amino acid sequences represented by SEQ ID NOS: 1 to 32 described above, or a repetitive structure of any of these amino acid sequences or a complex of these amino acid sequences.

Furthermore, the peptide may be an aluminum oxide affinity peptide containing one or more amino

acids selected from those of SEQ ID NOS: 30 to 32, characterized in that the one or more amino acids form a cyclic structure with an intramolecular disulfide binding in the sequence.

Furthermore, the DNA molecule according to the present invention is a DNA molecule characterized by including a DNA encoding an aluminum oxide affinity peptide chain containing the whole of at least one amino acid sequence or an amino acid sequence containing at least part thereof selected from the group consisting of amino acid sequences represented by SEQ ID NOS: 1 to 32 described above, or a repetitive structure of any of these amino acid sequences.

The vector according to the present invention is an expression vector capable of expressing the binding domain containing a peptide composed of the whole of at least one amino acid sequence or an amino acid sequence containing at least part thereof selected from the group consisting of amino acid sequences represented by SEQ ID NOS: 1 to 32 described above, or a repetitive structure of any of these amino acid sequences or a complex of these amino acid sequences and an organic substance containing a protein in at least part thereof as a fused product in a host cell, characterized by including a coupling gene containing a base sequence

made of a combination of a base sequence encoding an amino acid sequence of the protein included in the binding domain and a base sequence encoding an amino acid sequence of the protein included in the organic substance so as to encode a combination of the binding domain and the organic substance.

Hereinafter, the configuration of the organic substance-immobilized structure of the present invention and the manufacturing method thereof will be described in more detail.

In the organic substance-immobilized structure of the present invention, at least part of the surface of a substrate is provided with a region from which aluminum oxide is exposed. In the aluminum oxide-exposed region, the organic substance is immobilized in the organic substance-binding domain fused product through the binding domain containing a peptide composed of at least one or more amino acids, which is formed independently from the functional domain derived from the organic substance.

Figs. 1 to 3 each schematically show an example of the embodiment of the organic substance-immobilized structure in accordance with the present invention.

In the configuration shown in Fig. 1, a substrate is a flat substrate 11 the surface of which is provided with an aluminum oxide layer 12 as a

coating layer composed of a substrate containing aluminum oxide. The immobilization of the organic substance is carried out on the surface of the aluminum oxide layer 12 alternatively with the binding domain 15. On the other hand, the organic substance itself, which corresponds to the functional domain 14 being coupled with the binding domain 15, is able to perform weak physical adsorption non-selectively on the aluminum oxide layer 12. However, part of the binding domain 15, which specifically binds to the aluminum oxide, binds more dominantly. As a result, due to such a competing process, the organic substance itself corresponding to the functional domain 14 has a low frequency of causing physical adsorption on the surface of the substrate, and it is possible to reduce the non-specific weak adsorption by selecting the immobilization condition.

(Substrate)

As far as the surface of a substrate, for example a substrate, can be provided with a coating layer constructed of a substrate containing aluminum oxide, any of substrates made of various materials known in the art can be appropriately selected and used in accordance with the intended use. The substrate can be appropriately selected from substrates formed using metal materials such as iron, copper, gold, silver, and platinum, a polystyrene -

methyl methacrylate copolymer (PMMA), synthetic resin materials typified by polycarbonate (PC), semiconductor materials such as silicon, oxides of silica, sapphire, and so on, and ceramics materials, or complex materials made of combinations of two or more of these materials.

Variations of physical or chemical values caused in the vicinity of the organic substance of the present invention when the substrate is made of an electroconductive material or a complex material prepared by stacking layers of electroconductive layers. Alternatively, the substrate may be made of a translucent material to determine the variations caused in the vicinity of the organic substance of the present invention as, for example, a change in refractive index by means of an optical procedure.

In the case of a detecting element, a converting element, or the like using the structure of the present invention, it is more desirable to select the substrate in sufficient consideration of the matters described above.

(Aluminum oxide layer)

At least part of the surface of the substrate is configured such that the surface layer of aluminum oxide is exposed. When the thickness of the aluminum oxide layer 12 formed on the surface of the substrate is determined in the order of nanometers, it is

preferable to apply a gas-phase deposition method such as a CVD method more suitable to the thin film formation.

At first, for instance, an aluminum oxide layer is formed on the surface of a substrate by means of a CVD method. Then, in the atmosphere or by means of a heating or electrochemical procedure, an aluminum oxide layer can be obtained by facilitating the oxidation of the aluminum oxide layer formed on the surface of the substrate. In the case of applying the aluminum oxide layer-forming method, there is a need of selecting a substrate that does not cause an alteration or a degrease in characteristics of the substrate itself during the deposition process of the aluminum oxide layer by the CVD method or the oxidation process by the heating or electric procedure.

Furthermore, preliminarily, aluminum oxide material particles are formed and suspended in a dispersion solvent which is selected in consideration of the nature of the substrate material. Then, the suspension of aluminum oxide material particles is applied in a predetermined film thickness and if desired the dispersion solvent is removed by heating, allowing the formation of a layer containing particulate aluminum oxide.

In the case of using any procedure, if there is

a large difference in coefficient of thermal expansion between the aluminum oxide layer formed and the substrate, mechanical distortion may be induced in the aluminum oxide layer at the time of a heat treatment to cause peeling or warping of the aluminum oxide layer formed. In consideration of this fact, it is preferable to determine the thickness of the aluminum oxide layer in addition to the selection of the substrate material.

Furthermore, when the aluminum oxide layer 12 is provided as a porous material-coating structure, the specific surface area of the aluminum oxide layer 12 increases because of the presence of fine pores in the porous material. Thus, the total surface area available for the immobilization can be increased.

In addition, comparing with the immobilization of the organic substance on the flat substrate, the porous material-coating structure can be considered to have reduced reaction fields in the respective fine pores. For example, when the structure of the present invention is used as a target material capturing element or a converting element, the average moving distance between the target substance and the organic substance immobilized on the structure of the present invention can be reduced to a large extent, so an effect of increasing the reaction efficiency of molecular recognition can be

also expected.

Furthermore, by selecting the pore sizes of the fine pores, the target substance and other contaminate substances can be separated from each other on the basis of the pore sizes of the fine pores.

According to the present invention, when the aluminum oxide layer is provided as a porous material, it is preferable to properly select a suitable pore size so that the characteristics of the above porous material can be exerted, and also depending on the use of the element. In addition, it is preferable to adjust the thickness of the aluminum oxide layer in consideration of the strength of the aluminum oxide layer or the adhesiveness thereof to the substrate, while depending on the demanded characteristics of the adaptive element or the like.

A method of providing the aluminum oxide layer as the porous material layer can be selected from the methods known in the art. For example, an anodic oxidation process may be used for the formation of a porous aluminum oxide layer. The use of the anodic oxidation method allows the formation of the fine pores in the resulting aluminum oxide porous material in the order of nanometers. In addition, the pore size may be adjusted by regulation of an oxidation condition such as an applied voltage.

In Fig. 2 and Fig. 3, an example of the configuration of the aluminum oxide in the organic substance-immobilized structure of the present invention is represented by the respective schematic cross-sectional diagrams.

In Fig. 2, an example of the porous material of the aluminum oxide layer formed on the substrate material is represented by the schematic cross-sectional diagram.

Fig. 3 represents a schematic cross-sectional diagram of an example of a thin film of the aluminum oxide porous material.

(Organic substance)

In the present invention, the organic substance immobilized on the surface of the substrate can be properly selected depending on the intended use of the organic substance-immobilized substrate to be manufactured. As far as the organic substance used as a functional domain can be coupled with a binding domain containing a peptide constructed of at least one or more amino acids, the type of the organic substance used as a functional domain is not particularly limited. Each of various biological substances, which can be coupled with the binding domain containing a peptide composed of one or more amino acids, can be selected as an organic substance to be immobilized on the surface of the substrate.

Specific examples of a biological substance that can be selected as an organic substance to which the present invention is applicable include nucleic acid molecules, amino acids, peptides or proteins, and sugar chains and sugar chain-protein complexes. Of those, the peptides or proteins, and the sugar chains or sugar chain - protein complexes are more preferable.

The peptides include various peptides capable of specifically binding to the target substances and peptide hormones typified by insulin.

In addition, examples of protein molecules, which can be selective for the organic substance to which the present invention can be applied, include enzymes, antibodies, receptor molecules, and scaffolding protein molecules. Those known in the art can be used as the enzymes.

For example, in consideration of the target substance-detecting element for the organic substance-immobilized structure of the present invention, the enzymes include glucose dehydrogenase and glucose oxidase. In consideration of the target substance-converting element for the organic substance-immobilized structure of the present invention, a preferable enzyme is one that converts a substance provided as a raw material into the desired substance. Specific examples thereof include

converting enzymes known in the art, such as aminoacylase, lipase, and phosphodiesterase. Furthermore, various protein-refolding assisting proteins including GroEL referred to as molecular chaperones can be immobilized. The antibody molecules to which the present invention can be applied include immunoglobulin molecules collected by various kinds of methods, such as immune-antibody molecules produced as a result of immune reactions caused by the introduction of antigenic substances into test animals and recombinant antibody molecules obtained by partially or wholly altering the structures of the immune antibodies by means of genetic engineering.

The antibodies used in the present invention may be monoclonal or polyclonal antibodies. Those antibody molecules are included in any immunoglobulin class, and can be selected from, for example, human IgG, IgM, IgA, IgD, and IgE. Of those classes, the IgG-class antibody molecules can be used more preferably.

In addition to the immunoglobulin molecules, antibody fragment molecules, including Fab, Fab', and $F(ab')_2$, can be used. For example, the Fab fragment molecule is a fragment molecule almost identical to an antibody fragment molecule obtained by subjecting antibody globulin to papain digestion. $F(ab')_2$ is a

fragment molecule almost identical to an antibody fragment molecule obtained by subjecting antibody globulin to pepsin digestion.

Even though there is a method by which those antibody fragment molecules can be prepared by enzymatically or chemically decomposing antibody globulin, a method of recombinant production with genetic engineering can be also applied to most of the cases. Furthermore, a single-chain Fv (scFv), which is considered to be a genetically-engineered recombinant peptide having an antigen-recognizing ability by coupling a heavy chain (VH) with a light chain (VL), which constitute a variable region (Fv), an antigen recognition site in an immunoglobulin molecule, through a peptide constructed of several amino acids of the carboxyl end of one of them and of the amino end of the other.

The scaffolding proteins are proteins or cell-adhesion molecules which themselves cannot carry out functions of capturing, converting, and separating target substances, but are capable of binding substances having those functions such as enzymes and antibodies, so the scaffolding proteins can be selected from various known proteins and used.

When the functional domain 14 is the protein as described above and can be produced as a recombinant using a host cell, the binding domain 15 that

contains a peptide constructed of one or more amino acids coupled with the functional domain 14 may be configured as a fused protein in which their peptide chains are coupled with each other in line. In this case, a linker sequence having an appropriate number of amino acid residues may be inserted between the functional domain 14 portion and the binding domain 15 portion.

On the other hand, when the organic substance to which the present invention is applied has a sequence corresponding to an unknown protein, nucleic acid molecule, or sugar chain, the biological substance, the binding domain containing the peptide structure, or both of them are previously subjected to chemical modification/conversion such as introduction of a reactive functional group used for their combination as far as the modification and the conversion are within the scope of having no serious impact on their functions, allowing the production of a complex in which the substance and the domain are coupled with each other through chemical bonding. In particular, a binding domain fused product containing a biological substance - peptide structure can be formed by chemically binding between functional groups after previously subjecting the biological substance and/or the binding domain containing the peptide structure to chemical

modification/conversion such that the reactive functional groups which can be used for their combination will make a combination of: a maleimide group and a sulfonyl group (-SH); a succinimide group and an amino group; an isocyanate group and an amino group; a halogen and a hydroxy group; a halogen and a sulfonyl group (-SH); an epoxy group and an amino group; or an epoxy group and a sulfonyl group (-SH).

Furthermore, when the organic substance to which the present invention is applied is a lipid molecule, a binding domain complex containing a lipid - peptide structure is produced by: making the "binding domain" having a "hydrophobic peptide structure" containing a plurality of amino acids having free hydrophobic groups such as alanine, valine, leucine, isoleucine, methionine, tryptophan, and phenylalanine in addition to the aluminum oxide binding peptide structure, and then performing hydrophobic binding to the "hydrophobic peptide structure" of the lipid molecule, and may be used instead of the fused product.

(Binding domain)

In the organic substance-immobilized structure according to the present invention, the binding domain 15 used for immobilization to the surface of the aluminum oxide layer formed on the surface of the substrate can take advantage of a molecule containing

a peptide chain constructed of one or more amino acids having an ability to specifically bind to the aluminum oxide layer 12 or a protein containing an amino acid sequence of the peptide chain.

In a preferred embodiment, the binding domain 15 to be used in the present invention has an amino acid sequence constructed of one or more amino acids having an affinity to the aluminum oxide layer 12.

Preferred examples of the amino acid sequence having the affinity to the aluminum oxide layer, which is included in the binding domain, include an amino acid containing the whole of at least one amino acid sequence selected from the group consisting of amino acid sequences represented by SEQ ID NOS: 1 to 32 described above, or an amino acid sequence containing a part thereof. Furthermore, the amino acid sequence may be one containing a repetitive structure of the above amino acid sequence or a complex of plural sequences selected from the amino acid sequences. Besides, the amino acid sequence of any of SEQ ID NOS: 30 to 32 may form a cyclic structure with an intramolecular disulfide binding between cysteines being contained. Those amino acid sequences are those of peptides having affinities to aluminum oxide, which have been finally obtained as a result of concentrated studies of the inventors of the present invention.

The binding domain at least part of which has the peptide can be selectively bound through the aluminum oxide affinity peptide to the substrate having a surface from which aluminum oxide is exposed. In other words, the organic substance-immobilized structure of the present invention can be constructed by those aluminum oxide affinity peptides and the target substance-detecting element, converting element, and separating element using the above structure can obtain their desired characteristic features, respectively.

It can be expected that a higher affinity to aluminum oxide can be attained by forming a repetitive structure made of one or more amino acid sequences described above or a complex of two or more amino acid sequence described above. In addition, the formation of an intramolecular disulfide binding structurally stabilizes the binding domain containing the aluminum oxide affinity peptide and also improves the binding affinity and molecular orientation.

The aluminum oxide affinity peptide as described above may be one having an amino acid sequence defined by screening of a random peptide library or an amino acid sequence reasonably designed on the basis of chemical properties of the aluminum oxide layer.

Hereinafter, a screening method of a random

peptide library for acquiring an amino acid sequence having an affinity to the aluminum oxide layer will be described.

The random peptide libraries available in screening include random synthetic peptide libraries in which random peptides are chemically synthesized in soluble forms, solid-phase immobilized peptide libraries in which random peptides are synthesized on resin beads, peptide libraries in which DNA of random sequences chemically synthesized are biosynthesized in ribosomal cell-free systems, for example a phage display peptide library prepared by coupling a random synthetic gene with a gene for the N-terminal end of a surface protein of M13 phage (such as a gene III protein), and a random peptide library displayed using similar procedures by fusing a bacterial layer protein, Omp A (Francisco, 1993, Proc. Natl., Acad. Sci. USA, 90, 10444-10448 or Pistor and Hoborn,1989,KIin.Wochenschr., 66,110-116), PAL(Fuchs et al.,1991,Bio/Technology,9,1369-1372), Lamb(Charbit et al., 1988, Gene,70,181-189 and Bradbury et al.,1993,Bio/Technology,1565-1568), finbrin (Hedeg. Aard and KlemM.,1989,Gene,85,115-124 and Hofnung, 1991, Methods Cell Biol., 34,77-105), and an IgA protease-6 region (Klauser et al.,1990,EMBO J., 9,1991-1999).

As a procedure of screening an amino acid

sequence having an affinity to aluminum oxide using those random peptide libraries, when a chemical synthetic peptide library is used, a peptide library is brought into contact with (or adsorbed to) a support or a substrate such as a column support or a plate, a fine particle, or the like, which is made of aluminum oxide and represents the surface characteristics which are the same as or similar to those of the structure of the present invention, and then a peptide having no affinity to the aluminum oxide layer is removed by a washing step, followed by collecting a peptide binding to the aluminum oxide layer. After that, by using the Edman degradation method or the like, an amino acid sequence thereof can be determined.

On the other hand, when the phage display peptide library is used, a phage library that displays the above various peptides is added to and brought into contact with the surface of the above support, substrate covered with the aluminum oxide, or fine particle, followed by washing the non-specific binding phages out under selected washing conditions. After washing, the remaining phages are eluted with acid or the like and are then neutralized, followed by infecting *E. coli* bacteria to amplify the phage. Repeating such selection (panning) several times allows a plurality of clones each having an

affinity to the target aluminum oxide layer to be concentrated.

Here, for obtaining a single clone, colonies are formed on a culture plate in a state of being infected again with *E. coli*. Each of the respective single colonies is incubated in a liquid medium, and then phages that reside in the supernatant of the medium are purified by means of precipitation with polyethylene glycol or the like to collect phagemids. By analyzing the base sequence of the phagemids, an amino acid sequence of the peptide having an ability to bind to the objective aluminum oxide can be known.

A screening procedure using the phage display peptide library described above is able to concentrate phages that represent a peptide more strongly binding to the aluminum oxide layer among a wide variety of peptide display phage libraries (typically 10^9 or more), so that it can be preferably used for the purpose of the present invention.

An example of a method of constructing a phage display random peptide library involves coupling a gene for the N-terminal end of the surface protein (e.g., gene III protein) of M13 phage with a synthetic gene encoding a random amino acid sequence. The method may be one of those reported in Scott, J.K. and Smith, G.P.,Science Vol.249,386(1990), Cwirla, S.E. et al., Proc. Natl. Acad. Sci. USA Vol.87, 6378,

(1990), and so on. The size of the inserted gene is not particularly limited as far as a peptide can be stably expressed. However, it is preferable that the inserted library have a suitable length corresponding to 6 to 40 amino acids (corresponding to a molecular weight of about 600 to 4,000), preferably 7 to 18 amino acids for allowing the resulting library to include all of the random sequences and to have the affinity.

In addition, it is also possible to display, on a phage surface protein, a cyclic peptide having an affinity to aluminum oxide, in which two or more cysteines are provided in a peptide sequence which can be displayed on the phage surface and an intramolecular disulfide binding is formed between the cysteines. The amino acid sequence of the aluminum oxide affinity peptide obtained by screening of the phage display peptide library may be also constructed of a serial repetitive structure as described above. When two or more amino acid sequences are obtained, a sequence constructed of an appropriate combination of amino acid sequences, which are connected in series, of the whole or part of at least one amino acid sequence selected from the group consisting of those amino acid sequences may be used as an amino acid sequence having an affinity to the aluminum oxide layer. In this case, it is

preferable to provide an appropriate spacer sequence between two different amino acid sequences. The spacer sequence is preferably of about 3 to 400 amino acids, and also the spacer sequence may include any kind of amino acid. Most preferably, the spacer sequence is one that does not prevent the functions of the functional domain and does not prevent the binding of an organic substance to the aluminum oxide layer.

The amino acid sequences each having an affinity to aluminum oxide, which can be used in the present invention, are amino acid sequences determined by screening of a random peptide library, as well as amino acid sequences reasonably designed on the basis of the chemical properties of the aluminum oxide layer. A library may be constructed of those amino acid sequences, and thus an amino acid sequence having a higher affinity can be selected from the library using the screening method as described above.

A fused protein obtained by coupling the binding domain containing the amino acid sequence having the aluminum oxide affinity peptide constructed of one or more amino acids with a protein having desired characteristic features to be provided as a functional domain is one stably produced by constructing an expression vector such that a gene

encoding an amino acid sequence of the binding domain containing at least one or more aluminum oxide affinity peptides according to the present invention is inserted into the upstream or downstream of a gene encoding the functional domain, while their reading frames are coincident with each other.

Furthermore, when one or more amino acid linkers are provided between the binding domain and the functional domain, a base sequence that encodes the linker sequence can be inserted into a base sequence encoding the binding domain and the functional domain, while their reading frames are kept to be matched. Consequently, the aluminum oxide layer affinity portion can be expressed by coupling with the N- or C-terminal end of a protein being considered to be the functional domain 14. In addition, an appropriate liner sequence may be inserted so as to be expressed as a binding domain.

The linker sequence is preferably of about 3 to 400 amino acid, and also the linker sequence may include any kind of amino acids. Most preferably, the linker sequence is one that does not prevent the functions of a protein provided as the functional domain 14 and does not prevent the binding of the binding domain 15 to the aluminum oxide layer.

A promoter sequence used in the expression vector, an antibiotic resistance base sequence for

confirming transformation, and so on can be appropriately selected from those known in the art and used.

The immobilization of the fused protein thus obtained to the aluminum oxide layer is carried out through an amino acid sequence (hereinafter, referred to as an aluminum oxide layer affinity portion 15a) obtained by the screening procedure in the binding domain 15 which is fused with the functional domain 14 and then translated.

In the aluminum oxide layer 12 having a hydrophilic surface, the immobilization to the aluminum oxide layer 12 through the binding domain 15 can be strengthened by selecting, as the aluminum oxide layer affinity portion 15a to be fused with the functional domain 14 and to be translated, a sequence containing a number of amino acids having hydrophilic groups, particularly cationic residues or hydroxy groups, from the amino acid sequences.

A method of isolating and purifying a fused protein constructed by coupling the protein provided as the functional domain 14 and the binding domain containing the aluminum oxide layer affinity portion 15a can be any of methods as far as the method retains the activity of the protein provided as the functional domain.

Hereinafter, a method of manufacturing the

organic substance-immobilized structure of the present invention will be described.

A step of immobilizing an organic substance on the aluminum oxide layer 12 through a binding domain containing the aluminum oxide layer affinity portion 15a can be attained by bringing a fused product constructed of the organic substance and the binding domain into contact with the aluminum oxide layer 12 in an aqueous medium.

In the present invention, the composition of the aqueous medium used in the step of carrying out the immobilization through the binding domain may be one that does not prevent binding or converting reaction of the objective compound, which is performed by an organic substance to be immobilized, such as a biological substance. However, in order to skip the subsequent steps, the composition of the aqueous medium may be one capable of exerting a binding or converting reaction activity represented by the biological substance. Here, for example, a buffer may be used as the composition of the aqueous medium that exerts the activity. Examples of the buffer include general buffers used in biological reactions, such as an acetic acid buffer, a phosphoric acid buffer, a potassium phosphate buffer, a 3-(N-morpholino)propane-sulfonic acid (MOPS) buffer, an N-tris(hydroxymethyl)methyl-3-aminopropane

sulfonic acid (TAPS) buffer, a tris-HCl buffer, a glycine buffer, and a 2-(cyclohexylamino)ethane sulfonic acid (CHES) buffer, which are preferably used. For instance, when the biological substance is a PHA synthetic enzyme protein as described below, the concentration of a buffer used for exerting an enzymatic activity is generally in the range of 5 mM to 10 M, preferably in the range of 10 mM to 200 mM. In addition, pH is adjusted to 5.5 to 9.0, preferably 7.0 to 8.5.

The immobilization of the fused product constructed of the organic substance and the binding domain on the aluminum oxide layer 12 of the substrate's surface through the binding domain 15 can be attained by providing a liquid, in which a substrate on which the aluminum oxide layer 12 is mounted is immersed, as a solution where a fused product composed of the organic substance and the binding domain is dissolved in the aqueous medium so as to become a predetermined concentration. At this time, it is preferable to shake a reaction container or stir the contents thereof appropriately so as to allow the binding domain portion containing the aluminum oxide layer affinity portion 15a included in the fused product constructed of the organic substance and the binding domain to bind uniformly to the surface of the aluminum oxide layer.

In the above immobilization process, the composition of the aqueous medium used is preferably set in consideration of a change in electrical charges of the surface charges or hydrophobic property of the aluminum oxide layer affinity portion 15a contained in the binding domain and the aluminum oxide layer because those factors vary depending on a pH or a salt concentration of the aqueous medium. For example, the hydrophobicity of both of them can be increased by an increase in salt concentration.

In addition, it is also possible to set the composition of the solution suitable for the binding of the binding domain by investigating whether the aluminum oxide layer 12 is hydrophilic or hydrophobic by previously measuring the wetting angle of the solvent to the aluminum oxide layer 12 provided on the surface of the substrate. Furthermore, the binding amount of the aluminum oxide layer affinity portion 15a to the surface of the aluminum oxide layer 12 may be directly determined to set the composition of the solution. The determination of the binding amount may be carried out using, for example, a method by which a fused product solution constructed of an organic substance and the binding domain at known concentrations is added to a certain area of an aluminum oxide layer and subjected to immobilization treatment, and then the concentration

of the fused product constructed of the organic substance and the binding domain remaining in the solution is determined, followed by calculating a binding amount by a subtraction procedure.

Duration of the immobilization treatment for the biological substance is preferably in the range of 1 minute to 48 hours, more preferably in the range of 10 minutes to 3 hours. In general, it is not preferable to leave the substance standing or leave the substance for a much longer period because the desired functional activity of the immobilized biological substance is likely to lower.

[Examples]

Hereinafter, the present invention will be described in more detail by way of examples. In addition, those examples are illustrative of the best mode of the present invention. However, the present invention is not limited to configurations described in those examples.

In the examples described below, as for the organic substance immobilized structure, particularly the biological substance immobilized substrate of the present invention, a polyhydroxyalkanoate (PHA) synthetic enzyme protein is employed as a biological substance that corresponds to a functional domain and an aluminum oxide layer affinity peptide is employed as a binding domain. Then, a biological substance-

immobilized substrate, in which the fused product PHA synthetic enzyme protein constructed of the aluminum oxide layer affinity peptide being coupled with the N-terminal of the PHA synthetic enzyme protein through a linker sequence is immobilized on a substrate having a surface covered with an aluminum oxide layer, is exemplified for describing the configuration of the biological substance immobilized substrate and a manufacturing method therefor, specifically.

Furthermore, a method of obtaining an amino acid sequence of the aluminum oxide layer affinity peptide used as the binding domain will be described specifically.

Prior to those examples, in Reference Example 1, a method of producing a PHA synthetic enzyme protein as the biological substance corresponding to the functional domain by means of gene recombination, an enzymatic activity of the recombinant PHA synthetic enzyme protein, and a method of determining the enzymatic activity will be described in advance.

Furthermore, in the examples, for a biological substance immobilized substrate having a fused product type PHA synthetic enzyme protein immobilized on a substrate having a surface covered with an aluminum oxide layer, an enzymatic activity retained by the fused product type PHA synthetic enzyme

protein being immobilized can be examined through evaluation on the basis of an enzymatic activity of the recombinant PHA synthetic enzyme protein.

(Reference Example 1)

A transformant having an ability to produce a PHA synthetic enzyme and a transformant having an ability to produce a PHA synthetic enzyme by production of the PHA synthetic enzyme with recombination were manufactured by the following methods:

At first, strain YN2 (*Pseudomonas cichorii* YN2, FERM BP-7375) having an ability to produce a PHA synthetic enzyme was incubated overnight at 30°C in 100 ml of an LB medium (1% polypepton, 0.5% yeast extract, and 0.5% sodium chloride, pH 7.4), and then chromosomal DNA of the strain YN2 was isolated and collected by the method of Marmer et al. The chromosomal DNA thus obtained was completely digested with the restriction enzyme HindIII. A cloning vector used was pUC18 and cleaved by the restriction enzyme HindIII. The end of the product was subjected to a dephosphorization process (Molecular Cloning, 1, 572 (1989); Cold Spring Harbor Laboratory Press), and then a chromosomal DNA fragment completely digested with HindIII was coupled and inserted into a cleaved site (cloning site) of the vector using a DNA ligation kit Ver. II (Takara Shuzo

Co., Ltd.). By using the plasmid vector incorporated with the chromosomal DNA fragment, *E. coli* (*Escherichia coli*) strain HB101 was transformed to make a DNA library of the strain YN2.

Next, for selecting a DNA fragment containing a PHA synthetic enzyme gene originated from the strain YN2, a probe for colony hybridization was prepared. Oligonucleotides consisting of base sequences of SEQ ID NO: 37 and SEQ ID NO: 38 were synthesized, respectively (Amersham Pharmacia Biotech). Then, by using these two different origonucleotides as a pair of primers and the chromosomal DNA as a template, PCR amplification was carried out. A DNA fragment was isolated as PCR amplification product and then used as a probe for colony hybridization. The probe was labeled using a commercially-available alkaline phosophatase-labelling enzyme, AlkPhosDirect (Amersham Pharmacia Biotech). By using the resulting enzyme-labeled probe, A *E. coli* strain having a recombinant plasmid containing the objective PHA synthetic enzyme gene was selected from the chromosomal DNA library of the strain YN2 by means of a colony hybridization method. From the selected bacterial strain, the plasmid was collected by an alkaline process. Consequently, a DNA fragment containing a PHA synthetic enzyme gene originated from the strain YN2 was obtained.

(SEQ ID NO: 37) Base sequence of forward primer
5'—TGCTGGAACT GATCCAGTAC—3'
(SEQ ID NO: 38) Base sequence of reverse primer
5'—GGGTTGAGGA TGCTCTGGAT GTG—3'

The PHA synthetic enzyme gene DNA fragment obtained herein was incorporated by gene recombination in a vector pBBR 122 (Mo Bi Tec) containing a wide host range replication region which does not belong to an incompatible group of IncP, IncQ, or IncW. The recombinant plasmid was transformed into *Pseudomonas cichorii* strain YN2ml (PHA synthetic ability defective strain) by an electroporation process. The transformed strain YN2ml recovered its PHA synthetic ability and showed complementarity. Therefore, it was confirmed that the selected gene DNA fragment contains a PHA synthetic enzyme gene region which can be translated into a PHA synthetic enzyme at least in *Pseudomonas cichorii* strain YN2ml.

The base sequence of the respective DNA fragments containing the PHA synthetic enzyme gene originated from the strain YN2 was determined. As a result, it was confirmed that the defined base sequence includes two different base sequences represented by SEQ ID NO: 33 and SEQ ID NO: 34 encoding the respective peptide chains. Proteins composed of two different peptide chains encoded by

the two base sequences had PHA synthetic enzyme activities as described below, respectively. Thus, it was confirmed that the base sequences represented by SEQ ID NO: 33 and SEQ ID NO: 34 were PHA synthetic enzyme genes, respectively. That is, an amino acid sequence represented by SEQ ID NO: 35 was encoded by the base sequence of SEQ ID NO: 33, while an amino acid sequence represented by SEQ ID NO: 36 was encoded by the base sequence of SEQ ID NO: 34. The PHA synthetic ability could be also exerted from either of the proteins containing these two different amino acid sequences.

For the PHA synthetic enzyme gene having the base sequence represented by SEQ ID NO: 33, a full-length PHA synthetic enzyme gene was prepared again by carrying out PCR amplification using the chromosomal DNA as a template.

For the base sequence represented by SEQ ID NO: 34, an oligonucleotide (SEQ ID NO: 41) provided as an upstream primer and having a base sequence that is upstream of its initiation codon and an oligonucleotide (SEQ ID NO: 39) provided as a downstream primer and having a base sequence that is downstream of its terminal codon were designed and synthesized (Amersham Pharmacia Biotech). PCR amplification was carried out such that these two different oligonucleotides were used as a pair of

primers and the chromosomal DNA was used as a template, amplifying a full-length PHA synthetic enzyme gene (LA-PCR kit; Takara Shuzo Co., Ltd.).

(SEQ ID NO: 41) Base sequence of primer on upstream side

5'—GGACCAAGCT TCTCGTCTCA GGGCAATGG—3'

(SEQ ID NO: 39) Base sequence of primer on downstream side

5'—CGAGCAAGCT TGCTCCTACA GGTGAAGGC—3'

Similarly, for the PHA synthetic enzyme gene having the base sequence represented by SEQ ID NO: 34, PCR amplification was carried out using the chromosomal DNA as a template, re-preparing a full-length PHA synthetic enzyme gene. For the base sequence represented by SEQ ID: 34, an oligonucleotide (SEQ ID NO: 40) provided as an upstream primer and having a base sequence that is upstream of its initiation codon and an oligonucleotide (SEQ ID NO: 42) provided as a downstream primer and having a base sequence that is downstream of its terminal codon were designed and synthesized, respectively (Amersham Pharmacia Biotech). By using this oligonucleotide as a primer, PCR amplification was carried out, amplifying the full-strength of the PHA synthetic enzyme gene (LA-PCR kit; Takara Shuzo Co., Ltd.).

(SEQ ID NO: 40) Base sequence of primer on upstream

side

5'—GTATTAAGCT TGAAGACGAA GGAGTGTTG—3'

(SEQ ID NO: 42) Base sequence of primer on downstream side

5'—CATCCAAGCT TCTTATGATC GGGTCATGCC—3'

Next, two different PCR-amplified fragments, which contained the full-length PHA synthetic enzyme gene, obtained as described above were completely digested using the restriction enzyme HindIII, respectively. In addition, the expression vector pTrc99A was also cleaved by the restriction enzyme HindIII, followed by subjecting it to a dephosphorization process (Molecular Cloning, 1, 572 (1989); Cold Spring Harbor Laboratory Press). Then, each of the two different PCR-amplified fragments containing the full-length PHA synthetic enzyme gene, in which unnecessary base sequences were removed from both ends, was coupled with the HindIII-cleaved sites of the expression vector pTrc99A using a DNA ligation kit Ver. II (Takara Shuzo Co., Ltd.), making two different recombinant plasmids.

From the recombinant plasmids thus obtained, *E. coli* (*Escherichia coli* HB 101, Takara Shuzo, Co., Ltd.) was transformed by a calcium chloride method. The resulting recombinants were incubated and then the application of the recombinant plasmids was performed, followed by collecting the respective

recombinant plasmids. The recombinant plasmid that retains the full-length PHA synthetic enzyme gene DNA containing the base sequence of SEQ ID: 33 was referred to as pYN2-C1 (originated from SEQ ID: 37). Also, the recombinant plasmid that retains the full-length PHA synthetic enzyme gene DNA containing the base sequence of SEQ ID NO: 34 was referred to as pYN2-C2 (originated from SEQ ID NO: 38).

By using each of the recombinant plasmids pYN2-C1 and pYN2-C2, *E. coli* (*Escherichia coli* HB101fB fadB defective strain) was transformed by a calcium chloride method to obtain recombinant *E. coli* strains that retain the respective recombinant plasmids, a pYN2-C1 recombinant strain and a pYN2-C2 recombinant strain were obtained, respectively.

Each of the pYN2-C1 and pYN2-C2 recombinant strains was inoculated in 200 ml of an M9 medium containing 0.5% of yeast extract and 0.1% of octanoic acid, followed by incubating at 37°C while shaking at 125 strokes/min. After 24 hours passed, bacterial cells were collected by centrifugation and then plasmid DNA was collected by an ordinary method.

For the pYN2-C1, an oligonucleotide (SEQ ID NO: 43) provided as an upstream primer and an oligonucleotide (SEQ ID NO: 44) provided as a downstream primer were designed and synthesized, respectively (Amersham Pharmacia Biotech). PCR

amplification was carried out such that these two different oligonucleotides were used as a pair of primers and the pYN2-C1 was used as a template, resulting in a DNA as an amplified product having BamHI and SacI restriction sites on its upstream side and SpeI and XhoI restriction sites on its downstream side (LA-PCR kit; Takara Shuzo Co., Ltd.).

Upstream primer (SEQ ID NO: 43) :

5'—AGTGGATCCT CCGAGCTCAG TAACAAGAGT AACGATGAGT TGAAG —3'

Downstream primer (SEQ ID NO: 44) :

5'—ATACTCGAGA CTACTAGTCC GTTCGTGCAC GTACGTGCCT GGCGC —3'

Similarly, for the pYN2-C2, an oligonucleotide (SEQ ID NO: 45) provided as an upstream primer and an oligonucleotide (SEQ ID NO: 46) provided as a downstream primer were designed and synthesized, respectively (Amersham Pharmacia Biotech). PCR amplification was carried out such that these two different oligonucleotides were used as a pair of primers and the pYN2-C2 was used as a template, resulting in a DNA including a full-length PHA synthetic enzyme gene as an amplified product, which has a BamHI restriction site on its upstream side and an XhoI restriction site on its downstream side (LA-PCR kit; Takara Shuzo Co., Ltd.).

Upstream primer (SEQ ID NO: 45) :

5'—ATACTCGAGA CTACTAGTGC GCACGCGCAC GTAAGTCCCG GGCGC —3'

Downstream primer (SEQ ID NO: 46) :

5'—AGTGGATCCT CCGAGCTCCG CGATAAACCT GCGAGGGAGT CACTA —3'

The purified PCR-amplified products were digested with the restriction enzymes BamHI and XhoI and then inserted into the corresponding sites of plasmid pGEX-6P-1 (Amersham Pharmacia Biotech), respectively. By using these two different vectors (pGEX-C1 and pGEX-C2), *E. coli* (JM109) was transformed to obtain expression bacterial strains. The introduction of expression vectors into the respective bacterial cells was confirmed by checking the molecular weights of DNA fragments obtained by treating plasmid DNA prepared in large quantity using Miniprep (Wizard Minipreps DNA Purification Systems, manufactured by PROMEGA Co., Ltd.) with the restriction enzymes BamHI and XhoI. Each of the resulting expression bacterial cells was pre-cultured overnight in 10 ml of an LB-Amp medium, and then 0.1 ml of the culture was added to 10 ml of the LB-Amp medium, followed by incubating at 37°C while shaking at 170 rpm for 3 hours. Subsequently, IPTG (1 mM in final concentration) was added and further incubated at 37°C for 4 to 12 hours.

The IPTG-induced *E. coli* cells were centrifuged

(800xg, 2 minutes, 4°C) and collected, followed by re-suspending in 1/10 volume of PBS at 4°C. The bacterial cells were crushed by freeze-thawing, and sonication and then centrifuged (8000xg, 10 minutes, 4°C) to remove solid residual matters. By using SDS-PAGE, the presence of the objective protein being expressed (GST-fused protein) in a supernatant was confirmed. Then, the GST-fused protein being induced and expressed was purified using glutathione-sepharose 4B (Glutathion Sepharose 4B beads: manufactured by Amersham Pharmacia Biotech, Co., Ltd.).

The glutathion sepharose used was processed in advance to prevent non-specific adsorption. That is, the glutathion sepharoseu was washed three times with an equal volume of PBS (8,000 xg, 1 minute, 4°C), followed by adding an equal volume of a 4%-BSA-containing PBS to carry out treatment at 4°C for 1 hour. After the treatment, it was washed twice with an equal volume of PBS and then re-suspended in a 1/2-volume of PBS. Subsequently, 40 µl of pre-treated glutathion sepharose was added to 1 ml of a cell-free extract and gently stirred at 4°C. The stirring treatment allows the GST-fused proteins GST-YN2-C1 and GST-YN2-C2 to be adsorbed on the glutathion sepharose using the binding ability of the fusion partner GST.

After adsorption, the resultant was centrifuged (8,000 xg, 1 minute, 4°C) to collect glutathion sepharose, followed by washing three times with 400 µl of PBS. Subsequently, 40 µl of 10-mM glutathion was added and stirred at 4°C for 1 hour, eluting the adsorbed GST-fused protein. A supernatant containing the GST-fused protein was collected, followed by dialysis with respect to PBS to purify the GST-fused protein. After the purification, the SDS-PAGE confirmed that the product showed a single band.

Each 500-µg GST-fused protein was digested with PreScission protease (Amersham Pharmacia Biotech, 5U) and then a fusion partner GST portion at the N-terminal and a PHA synthetic enzyme protein at the C-terminal were separated from each other, followed by removing the protease and GST through a glutathione sepharose column. A flow-through fraction of the glutathione sepharose column was passed through a Sephadex G200 column being equilibrated with PBS, obtaining final purified products of the expression proteins YN2-C1 and YN2-C2, respectively. By using the SDS-PAGE, the expression proteins YN2-C1 and YN2-C2 being finally purified were confirmed to show single bands of 60.8 kDa and 61.5 kDa, respectively.

The activity of each purified enzyme protein was determined.

Activity measurement of the PHA synthetic

enzyme was carried out according to the following procedures based on an evaluating method including coloring CoA with 5,5'-dithio-bis-(2- nitrobenzoic acid) and measuring the amount of the CoA released in the process of converting 3-hydroxyacyl CoA provided as a substrate to PHA by polymerization with a catalytic action of the PHA synthetic enzyme.

Reagent 1:

Bovine serum albumin (manufactured by Sigma Co., Ltd.) was dissolved in 0.1 M tris-HCl buffer (pH 8.0) in an amount of 3.0 mg/mM.

Regent 2:

3'-hydroxyoctanoyl CoA was dissolved in 0.1 M tris-HCl buffer (pH 8.0) in an amount of 3 mg/ml.

Regent 3:

Trichloroacetic acid was dissolved in 0.1 M tris-HCl buffer (pH 8.0) in an amount of 10 mg/ml.

Regent 4:

5,5'-dithiobis-(2-nitrobenzoic acid) was dissolved in 0.1 M tris-HCl buffer (pH 8.0) in an amount of 2.0 mM.

A first reaction (PHA synthetic reaction): 100 µl of Regent 1 was added and mixed in 100 µl of a sample (enzyme) solution, and pre-incubated at 30°C for 1 minute, and the mixture was then added and mixed with 100 µl of Reagent 2 and pre-incubated at 30°C for 1 to 30 minutes, followed by terminating an

enzymatic reaction by the addition of Regent 3.

A second reaction (coloring reaction of free CoA): The first reaction solution that stopped the reaction was centrifuged (15,000 xg, 10 minutes) to collect a supernatant. Then, 500 µl of the supernatant was added with 500 µl of Reagent 4 and incubated at 30°C for 10 minutes, followed by measuring the absorbance at 412 nm to determine the level of the contained CoA.

Calculation of enzymatic activity: An amount of enzyme that causes the release of CoA in an amount of 1 µmol per minute is defined as 1 unit (U). In addition, the concentration of protein in the sample was measured using a micro-BCA protein quantitative assay reagent kit (manufactured by Pierce Chemical Co., Ltd.). The results of the activity measurement on each purified enzyme are shown in Table 1.

[Table 1]

PHA synthetic enzyme	Activity	Specific activity
YN2-C1	2.1 U/ml	4.1 U/mg protein
YN2-C2	1.5 U/ml	3.6 U/mg protein

Depending on the measured activities, the above enzyme solution was concentrated using an agent for concentrating a biological solution sample

("Mizubutori Kun" AB-1100, manufactured by ATTO Corporation), resulting in 10 U/ml of a purified enzyme solution.

In the examples described below, the PHA synthetic enzyme protein YN-C1 having a high specific activity will be used.

Example 1

Procurement of amino acid sequence having affinity to aluminum oxide nanoholes

1) Selection of aluminum oxide affinity phage by panning technique

(Step 1)

With 0.1% Tween-20/TBS buffer (50 mM tris-HCl, pH 7.5, 150 mM NaCl (hereinafter, referred as to TBST buffer)), 2×10^{11} pfu of the PhD.-12 phage display peptide library (NEW ENGLAND BIOLAB) was diluted to 0.5 ml to obtain a library suspension.

(Step 2)

For procurement of the amino acid sequence, 0.5 ml of the above library suspension was added into one well of a flat-bottomed 24-well titer plate in which one aluminum oxide membrane (60 μm in thickness, 13 mm in diameter, pore size 0.2 μm, trade name: Anodisc Membrane, manufactured by Whatman) was placed, and left to stand at 25°C for 30 minutes.

(Step 3)

The supernatant was discarded and the Anodisc

Membrane was washed ten times with 2 ml of TBST buffer within the above well.

(Step 4)

After 0.5 ml of elution buffer (0.2M Glycine-HCl (pH 2.2), 1 mg/ml BSA) was added to the Anodisc Membrane which had been already washed, and then gently shaken for 10 minutes, the supernatant was transferred into another well in the microtiter plate. To the dispensed supernatant, 75 µl of 1 M tris-HCl (pH 9.1) was added for neutralization to obtain a phage eluted from the Anodisc Membrane.

(Step 5)

The eluted phage was infected with *E. coli* ER2537 (manufactured by NEW ENGLAND BIOLAB) at the early stage of logarithmic growth phase and amplified according to the following procedures.

Following infection, the *E. coli* was cultured at 37 °C for 4.5 hours. Subsequently, by centrifugation, the phage was separated from the *E. coli* and precipitated from the supernatant by polyethyleneglycol to be purified. The phage which had been amplified and purified was suspended into TBS buffer. The above phage suspension was infected with the *E. coli* in appropriate dilution series, thereby measuring its titer.

(Step 6)

For the phage contained in the suspension which

had been primary screened to the Anodisc Membrane, the screening procedure in Step 1 to Step 5 described above was additionally repeated four times. However, for the secondary and subsequent screening, the washing condition in Step 3 was made more rigorous by increasing the concentration of Tween-20 in TBST buffer utilized for washing to 0.5% (hereinafter, 0.5% TBST buffer) to sort out the phage showing a higher affinity to the Anodisc Membrane. In addition, for the tertiary (the second time) and subsequent screening, the phage separated from the Anodisc Membrane by washing in Step 3 was applied to the same procedure and its titer was measured. This separated phage would be used as control.

Table 2 shows the titer of the phages eluted from the Anodisc Membrane in each time of the primary screening to the fifth screening.

[Table 2]

Titer of phage eluted in each time of screenings

	Stock Solution (A)	Control Binding (B)	Anodisc Membrane Binding (C)	C/A	C/B
1st time	2.0×10^{11}		4.7×10^{3}	2.4×10^{-8}	
2nd time	2.0×10^{11}	5.3×10^{2}	1.8×10^{3}	9.0×10^{-9}	3.4
3rd time	2.0×10^{11}	8.0×10^{1}	7.0×10^{3}	3.5×10^{-8}	8.8×10^{1}
4th time	2.0×10^{11}	2.0×10^{1}	1.8×10^{4}	9.0×10^{-8}	9.0×10^{2}
5th time	2.0×10^{11}	1.0	3.5×10^{4}	1.8×10^{-7}	3.5×10^{4}

(Units of A, B, and C = pfu/μl)

The phage eluted in the final screening step, which was sorted out in the above screening procedure, was cloned by its infection with a large excess of *E. coli*.

After each of the separated clones was infected with *E. coli* and amplified, ssDNA was prepared from the phages of each clone and the base sequence in the random region was decoded, thereby obtaining the phages of 51 clones having high affinities to the Anodisc Membrane.

The obtained phages of 51 clones were evaluated for affinity to aluminum oxide by the phage ELISA. In addition, the DNA sequences encoding each phage-displayed peptide portion were analyzed to determine the amino acid sequence of the peptide showing the binding ability to aluminum oxide.

2) Evaluation of aluminum oxide affinity by phage ELISA

(Step 1)

For the phage suspensions of individual clones of the above 51 clones which had been sorted out in screening described above, 2×10^{11} pfu equivalents of these suspensions were diluted with 0.5% TBST buffer to be brought to 0.5 ml.

(Step 2)

All of the above phage suspensions were each added to one well in a flat-bottomed 24-well titer

plate in which one Anodisc Membrane was placed, and were left to stand at 25°C for 30 minutes.

(Step 3)

The supernatant was discarded and the Anodisc Membrane was washed ten times with 2 ml of 0.5% TBST buffer within the above well.

(Step 4)

To the Anodisc Membrane in the above well which had been already washed, 0.5 ml of an HRP binding anti-M13 antibody solution (1µl of anti-M13 antibody (manufactured by NEW ENGLAND BIOLAB) was suspended in 10 ml of TBST) was added, and the whole was gently shaken for 60 minutes. Subsequently, the supernatant was discarded and the mixture was washed 5 times by repeating the washing procedure with 2 ml of 0.5% TBST buffer.

(Step 5)

The treatment was applied to the phage bound on the Anodisc Membrane, in which this phage was reacted with the above HRP binding anti-M13 antibody. To the well in which this treated Anodisc Membrane was placed, 0.5 ml of Detection Reagent 1 (Amersham Pharmacia, #RPN2209) was added.

Furthermore, 0.5 ml of Detection Reagent 2 (Amersham Pharmacia #RPN2209) was added thereto. After a lapse of three minutes, the emission intensity from luminol at 420 nm generated by the

effect of the labeled enzyme HRP in the HRP binding anti-M13 antibody was measured.

The result of evaluation for each clone is shown in Table 3. I_{420} indicates the emission intensity at 420 nm.

[Table 3]

Result of evaluation for aluminum oxide affinity by phage ELISA

Clone No	1	2	3	4	5	6	7	8
I_{420}	0.358	0.524	0.413	0.256	0.482	0.563	0.641	0.240

Clone No	9	10	11	12	13	14	15	16
I_{420}	0.269	0.462	0.324	0.650	0.470	0.584	0.419	0.581

Clone No	17	18	19	20	21	22	23	24
I_{420}	0.265	0.741	0.623	0.491	0.489	0.612	0.444	0.523

Clone No	25	26	27	28	29	30	31	32
I_{420}	0.223	0.551	0.542	0.469	0.378	0.380	0.264	0.701

Clone No	33	34	35	36	37	38	39	40
I_{420}	0.610	0.528	0.290	0.300	0.314	0.268	0.701	0.467

Clone No	41	42	43	44	45	46	47	48
I_{420}	0.345	0.516	0.410	0.432	0.489	0.236	0.521	0.584

Clone No	49	50	51
I_{420}	0.642	0.513	0.498

The emission intensity was 0.001, which was observed when the phage was not mixed with a solution brought into contact with the Anodisc Membrane in Step 2 in the above phage ELISA measurement system (control).

The foregoing evaluation confirmed that any of the peptides which 51 obtained phage clones displayed had an affinity to aluminum oxide.

3) Amino acid sequence showing binding ability to aluminum oxide

By comparison with the amino acid sequences of the random peptide display regions of each phage from the result of the DNA sequence analysis of the above phages, the amino acid sequences estimated to participate in an affinity to aluminum oxide were identified for the 51 phage clones sorted out. Table 4 shows the identified amino acid sequences showing affinities to aluminum oxide and their incidence.

[Table 4]

Identified amino acid sequence and Incidence

Identified amino acid sequence	Number (A)	Incidence (A/51)
Val-Tyr-Ala-Asn-Gln-Thr-Pro-Pro-Ser-Lys-Ala-Arg (Sequence No: 1)	11	0.22
Gln-Ser-Ser-Ile-Thr-Thr-Arg-Asn-Pro-Phe-Met-Thr (Sequence No: 2)	6	0.12
Phe-Met-Asn-His-His-Pro-Asn-Ser-Gln-Gln-Tyr-His (Sequence No: 3)	4	0.08
Gln-Tyr-Thr-Ser-Ser-Gly-Ile-Ile-Thr-Ser-Ser-Ala (Sequence No: 4)	3	0.06
His-His-His-Pro-Glu-Asn-Leu-Asp-Ser-Thr-Phe-Gln (Sequence No: 5)	3	0.06
Gln-Pro-His-Met-His-Arg-Ser-Ser-His-Gln-Asp-Gly (Sequence No: 6)	1	0.02
Asn-Thr-Thr-Met-Gly-Pro-Met-Ser-Pro-His-Ser-Gln (Sequence No: 7)	1	0.02
Ala-Ala-His-Phe-Glu-Pro-Gln-Thr-Met-Pro-Met-Ile (Sequence No: 8)	1	0.02
Asp-His-Gln-Leu-His-Arg-Pro-Pro-His-Met-Met-Arg (Sequence No: 9)	1	0.02
Val-Ser-Arg-His-Gln-Ser-Trp-His-Pro-His-Asp-Leu (Sequence No: 10)	1	0.02
Met-Met-Gln-Arg-Asp-His-His-Gln-His-Asn-Ala-Gln (Sequence No: 11)	1	0.02
Val-Thr-Leu-His-Thr-Val-Asp-His-Ala-Pro-Gln-Asp (Sequence No: 12)	1	0.02
Ser-Val-Ser-Val-Gly-Met-Lys-Pro-Ser-Pro-Arg-Pro (Sequence No: 13)	1	0.02
His-Leu-Gln-Ser-Met-Lys-Pro-Arg-Thr-His-Val-Leu (Sequence No: 14)	1	0.02
Ile-Pro-Asn-Ala-Glu-Thr-Leu-Arg-Gln-Pro-Ala-Arg (Sequence No: 15)	1	0.02
Val-Gly-Val-Ile-Ser-Ser-Trp-His-Pro-His-Asp-Leu (Sequence No: 16)	1	0.02
Thr-Val-Pro-Ile-Tyr-Asn-Thr-Gly-Ile-Leu-Pro-Thr (Sequence No: 17)	1	0.02
Tyr-Thr-Met-His-His-Gly-Ser-Thr-Phe-Met-Arg-Arg (Sequence No: 18)	1	0.02
Ser-Met-Met-His-Val-Asn-Ile-Arg-Leu-Gly-Ile-Leu (Sequence No: 19)	1	0.02
Ala-Pro-Met-His-His-Met-Lys-Ser-Leu-Tyr-Arg-Ala (Sequence No: 20)	1	0.02
Met-Met-Gln-Arg-Asp-His-His-Gln-His-Met-Arg-Arg (Sequence No: 21)	1	0.02
Met-Lys-Thr-His-His-Gly-Asn-Asn-Ala-Val-Phe-Leu (Sequence No: 22)	1	0.02

Leu-Glu-Pro-Leu-Pro-His-Thr-Pro-Arg-Met-Tyr-Ala (Sequence No: 23)	1	0.02
Gln-Leu-Tyr-Glu-Pro-Asp-Ser-Gly-Pro-Trp-Ala-Pro (Sequence No: 24)	1	0.02
Trp-Met-Thr-Lys-Met-Pro-Thr-Thr-His-Thr-Arg-Tyr (Sequence No: 25)	1	0.02
His-His-Pro-Met-Tyr-Ser-Met-Thr-Arg-Ala-Leu-Pro (Sequence No: 26)	1	0.02
Gly-Ser-Ala-His-Ser-Arg-Asn-Asp-Ala-Ala-Pro-Val (Sequence No: 27)	1	0.02
His-Ser-Pro-Leu-Met-Gln-Tyr-His-Met-Ser-Gly-Thr (Sequence No: 28)	1	0.02
Thr-Ala-His-Met-Thr-Met-Pro-Ser-Arg-Phe-Leu-Pro (Sequence No: 29)	1	0.02

Example 2

Procurement of cyclic amino acid sequence having affinity to aluminum oxide

1) Selection of aluminum oxide affinity phage by panning technique

(Step 1)

With 0.1% Tween-20/TBS buffer (50 mM tris-HCl, pH 7.5, 150 mM NaCl (hereinafter, referred as to TBST buffer)), 2 x 10^{11} pfu of the PhD.-C7C phage display peptide library (NEW ENGLAND BIOLAB) was diluted to 0.5 ml to obtain a library suspension.

(Step 2)

For procurement of the amino acid sequence, 0.5 ml of the library suspension was added into one well of a flat-bottomed 24-well titer plate in which one aluminum oxide membrane (60 μm in thickness, 13 mm in diameter, pore size 0.2 μm, Anodisc Membrane, manufactured by Whatman) was placed, and left to

stand at 25°C for 30 minutes.

(Step 3)

The supernatant was discarded and the Anodisc Membrane was washed ten times with 2 ml of TBST buffer within the above well.

(Step 4)

After 0.5 ml of elution buffer (0.2M Glycine-HCl (pH 2.2), 1 mg/ml BSA) was added to the Anodisc Membrane which had been already washed, and then gently shaken for 10 minutes, the supernatant was transferred into another well in the microtiter plate. To the dispensed supernatant, 75 µl of 1 M tris-HCl (pH 9.1) was added for neutralization to obtain a phage eluted from the Anodisc Membrane.

(Step 5)

The eluted phage was infected with *E. coli* ER2537 (manufactured by NEW ENGLAND BIOLAB) at the early stage of logarithmic growth phase and amplified according to the following procedures.

Following infection, the *E. coli* was cultured at 37°C for 4.5 hours. Subsequently, by centrifugation, the phage was separated from the *E. coli* and precipitated from the supernatant by polyethyleneglycol to be purified. The phage which had been amplified and purified was suspended into TBS buffer. The phage suspension was infected with the *E. coli* in appropriate dilution series, thereby

measuring its titer.

(Step 6)

With respect to the affinity of the peptide which the above phage displayed to the Anodisc Membrane, for the phage contained in the suspension which had been primary screened, the screening procedure in Step 1 to Step 5 described above was additionally repeated three times. However, for the secondary and subsequent screening, the washing condition in Step 3 was made more rigorous by increasing the concentration of Tween-20 in TBST buffer utilized for washing to 0.5% (hereinafter, 0.5% TBST buffer) to sort out the phage showing a higher affinity to the Anodisc Membrane. In addition, for the tertiary (the second time) and subsequent screening, the phage separated from the Anodisc Membrane by washing in the above Step 3 was applied to the same procedure and its titer was measured. This separated phage would be used as control.

Table 5 shows the titer of the phages eluted from the Anodisc Membrane in each time of the primary screening to the forth screening.

[Table 5]

Titer of Phage eluted in each time of screenings

	Stock Solution (A)	Control Binding (B)	Anodisc Membrane Binding (C)	C/A	C/B
1st time	2.0×10^{11}		1.1×10^{2}	5.5×10^{-10}	
2nd time	2.0×10^{11}	1.2×10^{1}	4.6×10^{1}	2.3×10^{-10}	4
3rd time	2.0×10^{11}	3.0	1.5×10^{2}	7.5×10^{-10}	5.0×10^{1}
4th time	2.0×10^{11}	1.0	1.0×10^{3}	5.0×10^{-9}	1.0×10^{3}

(Units of A, B, and C = pfu/μl)

The phage eluted in the final screening step, which was sorted out in the above screening procedure, was cloned by its infection with a large excess of *E. coli*.

After each of the separated clones was infected with *E. coli* and amplified, ssDNA was prepared from the phages of each clones and the base sequence in the random region was decoded, thereby obtaining the phages of 11 clones having high affinities to the Anodisc Membrane.

The obtained phages of 11 clones were evaluated for affinity to aluminum oxide by the phage ELISA. In addition, the DNA sequences encoding each phage-displayed peptide portion were analyzed to determine the amino acid sequence of the peptide showing the

binding ability to aluminum oxide.

2) Evaluation of aluminum oxide affinity by phage ELISA

(Step 1)

For the phage suspensions of individual clones of the above 11 clones which had been sorted out from the PhD.-C7C phage display peptide library (NEW ENGLAND BIOLAB), 2×10^{11} pfu equivalents of these suspensions were diluted with 0.5% TBST buffer to be brought to 0.5 ml.

(Step 2)

All of the phage suspensions were each added to one well in a flat-bottomed 24-well titer plate in which one Anodisc Membrane was placed, and were left to stand at 25°C for 30 minutes.

(Step 3)

The supernatant was discarded and the Anodisc Membrane was washed ten times with 2 ml of 0.5% TBST buffer within the above well.

(Step 4)

To the Anodisc Membrane in the above well which had been already washed, 0.5 ml of an HRP binding anti-M13 antibody solution (1µl of anti-M13 antibody (manufactured by NEW ENGLAND BIOLAB) was suspended in 10 ml of TBST) was added, and the whole was gently shaken for 60 minutes. Subsequently, the supernatant was discarded and the mixture was washed 5 times by

repeating the washing procedure with 2 ml of 0.5% TBST buffer.

(Step 5)

The treatment was applied to the phage bound on the Anodisc Membrane, in which this phage was reacted with the above HRP binding anti-M13 antibody. To the well in which this treated Anodisc Membrane was placed, 0.5 ml of Detection Reagent 1 (Amersham Pharmacia, #RPN2209) was added.

Furthermore, 0.5 ml of Detection Reagent 2 (Amersham Pharmacia #RPN2209) was added thereto. After a lapse of three minutes, the emission intensity from luminol at 420 nm generated by the effect of the labeled enzyme HRP in the HRP binding anti-M13 antibody was measured.

The result of evaluation for each clone is shown in Table 6. I_{420} indicates the emission intensity at 420 nm.

[Table 6]

Result of evaluation for aluminum oxide affinity by phage ELISA

Clone No	1	2	3	4	5	6	7	8
I_{420}	0.508	0.354	0.224	0.321	0.432	0.579	0.241	0.249

Clone No	9	10	11
I_{420}	0.235	0.198	0.364

The emission intensity was 0.001, which was observed when the phage was not mixed with a solution brought into contact with the Anodisc Membrane in Step 2 in the above phage ELISA measurement system (control).

The foregoing evaluation confirmed that any of the peptides which 11 obtained phage clones displayed had an affinity to aluminum oxide.

3) Amino acid sequence showing binding ability to aluminum oxide

By comparison with the amino acid sequences of the random peptide display regions of each phage from the result of the DNA sequence analysis of the phages, the amino acid sequences estimated to participate in an affinity to aluminum oxide were identified for the 51 phage clones sorted out. Table 7 shows the identified amino acid sequences showing affinities to aluminum oxide and their incidence.

[Table 7]

Identified amino acid sequence and Incidence

Identified amino acid sequence	Number (A)	Incidence (A/15)
Ala-Cys-Pro-Pro-Thr-Gln-Ser-Arg-Tyr-Cys (Sequence No: 30)	7	0.64
Ala-Cys-Asn-Gly-Met-Leu-Ala-Phe-Gln-Cys (Sequence No: 31)	3	0.27
Ala-Cys-Thr-Pro-Lys-Pro-Gly-Lys-His-Cys (Sequence No: 32)	1	0.09

Example 3

Aluminum oxide affinity peptide-fused PHA synthetic enzyme

1) Production of DNA fragment encoding aluminum oxide affinity peptide-fused PHA synthetic enzyme

An *E. coli* expression vector expressing the anodisc membrane binding peptide-PHA synthetic enzyme fused product which was produced by fusing the amino acid sequence showing an affinity to the above anodisc membrane, Val-Tyr-Ala-Asn-Gln-Thr-Pro-Pro-Ser-Lys-Ala-Arg (SEQ ID NO: 1), to the N-terminal of the PHA synthetic enzyme through the linker sequence GGGS was constructed as follows.

The DNA fragment encoding this anodisc membrane binding peptide and the linker sequence portion was created as double-stranded DNA and ligated into the appropriate restriction cleavage sites (BamHI and SacI) of the pGEX-C1 plasmid for expressing the fused protein GST-YN2-C1. In this case, the ends of two synthesized oligonucleotides O1 (5'-GATCCGTTTATGCGAATCAGACTCCGCCTTCTAAGGCGCGGGGTGGAGGTTCGGAGCT-3', SEQ ID NO: 47) and O2 (5'-CGAACCTCCACCCCGCGCCTTAGAAGGCGGAGTCTGATTCGCATAAAC-3', SEQ ID NO: 4) were phosphorylated using T4 polynucleotide kinase (manufactured by Gibco) according to the manufacturer's instruction. Subsequently, two equimolar synthesized DNAs were mixed and heated at 80°C for 5 minutes, followed by

slow cooling to room temperature, thereby forming the double-stranded DNA fragment. The formed double-stranded DNA fragment was directly used for subsequent cloning.

2) Gene transfer and expression and purification of fused product

The plasmid pGEX-C1 created in Reference Example 1 was digested with the restriction enzymes BamHI and SacI, into which in turn the above double-stranded DNA fragment was inserted. *E. coli* (JM109) was transformed with this vector to obtain a strain for expression. The confirmation of the introduction of the expression vector into each strain was carried out by determining the base sequence inserted between BamHI and SacI sites of the restriction enzymes by the sequencing of the plasmid DNA as a template which had been prepared using Miniprep (Wizard Minipreps DNA Purification Systems, manufactured by PROMEGA), with pGEX 5' Sequencing Primer (manufactured by Amersham Pharmacia Biotech). After the resulting strain for expression was pre-cultured overnight in 10 ml of LB-Amp medium, 0.1 ml of that cultured product was added to 10 ml of LB-Amp medium and cultured while being stirred at 37°C for 3 hours at 170 rpm. IPTG (final concentration 1mM) was then added thereto and the culture was continued at 37°C for 4-12 hours.

IPTG-derived *E. coli* was harvested (8000 xg, 2 min., 4°C) and resuspended into 1/10 volume of 4°C PBS. The fungus cellswas ruptured by freeze-thaw and sonication and centrifuged (8000 xg, 10 min., 4°C) to remove solid impurities. The expressed protein of interest was confirmed to exist in the supernatant by SDS-PAGE. Subsequently, from the supernatant, the GST-fused protein which had been derived and expressed was purified with Glutathion Sepharose 4B beads (manufactured by Amersham Pharmacia Biotech).

It is noted that the GST-fused protein which is derived and expressed is the fused protein GST-A01-GS-YN2-C1 into which the peptide chain is inserted where the amino acid sequence of the above SEQ ID NO: 1 and the linker sequence GGGS are coupled between the C-terminal of the GST protein as a fusion partner and the N-terminal of the PHA synthetic enzyme protein YN2-C1.

The glutathione sepharose used was previously treated for suppressing nonspecific absorption. That is, after the glutathione sepharose was washed (8000 xg, 1 min., 4°C) three times in the equal volume of PBS, the equal volume of 4% BSA-containing PBS was added thereto and the whole was treated at 4°C for 1 hour. Following the treatment, the glutathione sepharose was washed twice in the equal volume of PBS and resuspended into 1/2 volume of PBS. 40 μL of the

glutathione sepharose pretreated was added to 1 mL of a cell-free extract (supernatant) and the whole was gently stirred at 4°C. By this stirring, the fused protein GST-A01-GS-YN2-C1 was absorbed to the glutathione sepharose.

After the absorption, the glutathione sepharose was collected by centrifugation (8000 xg, 1 min., 4°C) and washed three times in 400 µl of PBS. Subsequently, 40 µl of 10 mM glutathione was added and the whole was stirred at 4°C for 1 hour to elute the fused protein which was absorbed. The supernatant containing the fused protein was collected by centrifugation (8000 xg, 2 min., 4°C) and dialyzed against PBS to purify the GST-fused protein. Following the purification, SDS-PAGE showed that the purified product showed a single band.

With PreScission protease (Amersham Pharmacia Biotech, 5U), 500 µg of each GST-fused protein was digested and the fusion partner GST portion at the N-terminal was separated by cleavage. This solution was loaded onto a glutathione sepharose column to remove protease and GST. The flow-through fraction of the glutathione sepharose column was further loaded onto the Sephadex G200 column equilibrated with PBS to obtain, as a final purified product, the peptide-fused protein A01-YN2-C1. SDS-PAGE showed that the expressed protein A01-GS-YN2-C1 which had

been already finally purified showed a single band.

The enzymatic activity of the resulting expressed protein A01-GS-YN2-C1 which had been already finally purified was measured by the method described in the above Reference Example 2. Moreover, the concentration of the protein in the sample was measured with the Micro BCA Protein Quantification Reagent Kit (manufactured by Pierce Chemical). The enzymatic activity of the sample was 1.9 U/ml with the specific activity of 4.0 U/mg protein. The enzymatic solution which had been already finally purified was concentrated using a biological solution sample concentrating agent (Mizubutorikun AB-1100, manufactured by ATTO Co., LTD.) to obtain 10 U/ml of the purified enzymatic solution.

3) Affinity evaluation for aluminum oxide particle of aluminum oxide affinity peptide-fused protein

The aluminum oxide particle AW40-74 (manufactured by Micron) was suspended into 0.1% Tween-20/TBS buffer so as to be 0.5% (w/v). In a centrifuge tube made from Teflon, 10 ml of this suspension was placed and 0.5 U equivalents of the peptide-fused PHA synthetic enzyme A01-GS-YN2-C1 prepared in Example 2 or the PHA synthetic enzyme YN2-C1 prepared in Reference Example 2 was added thereto and the whole was shaken at room temperature for 30 minutes. By the centrifugation procedure

(10,000 xg, 4°C, 10 min.), the AW40-74 particle as a precipitate was collected and separated from the supernatant containing the enzyme protein which had not been bound to the AW40-74. The AW40-74 which had been collected as a precipitate fraction was suspended again into TBS buffer containing 0.1% Tween-20 and centrifuged. The AW40-74 was washed by repeating the procedure of collecting the precipitate fraction. Table 8 shows the result of the enzymatic activity of the washed AW40-74 suspension measured by the measuring method described in the above Reference Example 2.

[Table 8]

Enzymatic activity of each PHA synthetic enzyme protein bound on aluminum oxide particle

PHA synthetic enzyme complex	Enzymatic activity (U)
A 01-GS-YN2-C1	0.11
YN 2-C1	0.01

Similarly, for thirty-one aluminum oxide affinity sequences shown in SEQ ID NO: 2 to SEQ ID NO: 32, the aluminum oxide particle-PHA synthetic enzyme complexes: A02-GS-YN2-C1 to A32-GS-YN2-C1 were prepared in the same manner as in Example 2 using synthesized oligonucleotides shown in SEQ ID NO: 49 to SEQ ID NO: 110, and the enzymatic activity of each

PHA synthetic enzyme bound to the aluminum oxide particle was measured in the same way in order to evaluating the binding ability to the aluminum oxide particle. The result of measurement is shown in Table 9.

[Table 9]

Enzymatic activity of each PHA synthetic enzyme protein bound on aluminum oxide particle

PHA synthetic enzyme complex	Enzymatic activity (U)
A02-GS-YN2-C1	0.09
A03-GS-YN2-C1	0.08
A04-GS-YN2-C1	0.08
A05-GS-YN2-C1	0.08
A06-GS-YN2-C1	0.09
A07-GS-YN2-C1	0.07
A08-GS-YN2-C1	0.06
A09-GS-YN2-C1	0.07
A10-GS-YN2-C1	0.08
A11-GS-YN2-C1	0.07
A12-GS-YN2-C1	0.07
A13-GS-YN2-C1	0.06
A14-GS-YN2-C1	0.08
A15-GS-YN2-C1	0.08
A16-GS-YN2-C1	0.07
A17-GS-YN2-C1	0.06
A18-GS-YN2-C1	0.06
A19-GS-YN2-C1	0.07
A20-GS-YN2-C1	0.09
A21-GS-YN2-C1	0.08
A22-GS-YN2-C1	0.06
A23-GS-YN2-C1	0.08
A24-GS-YN2-C1	0.05
A25-GS-YN2-C1	0.06
A26-GS-YN2-C1	0.07
A27-GS-YN2-C1	0.07
A28-GS-YN2-C1	0.07
A29-GS-YN2-C1	0.06
A30-GS-YN2-C1	0.10
A31-GS-YN2-C1	0.08
A32-GS-YN2-C1	0.05
YN2-C1	0.01

Comparison to the control enzyme protein YN2-C1 confirmed that the enzyme proteins A01-GS-YN2-C1 and A30-GS-YN2-C1 fusing, at the N-terminal, the peptide chain of the amino acid sequence having the binding ability to aluminum oxide were observed to have higher enzymatic activity and that the enzyme protein was allowed to be effectively immobilized on the surface of the substrate composed of the aluminum oxide through the peptide chain of the amino acid sequence having the binding ability to the aluminum oxide, which was fused at the N-terminal.

Example 4

Production of aluminum oxide affinity peptide-fused horseradish peroxidase (HPR)

An *E. coli* expression vector expressing the anodisc membrane binding peptide-HPR fused protein which is produced by fusing the amino acid sequence showing an affinity to the anodisc membrane, Val-Tyr-Ala-Asn-Gln-Thr-Pro-Pro-Ser-Lys-Ala-Arg (SEQ ID NO: 1), to the N-terminal of the HPR through the linker sequence GGGS is constructed as follows.

1) Production of DNA fragment encoding aluminum oxide affinity peptide-fused HPR and preparation of primer.

At first, the double-stranded DNA encoding the above anodisc membrane binding peptide-fused protein-linker sequence (GGGS)-HPR (A01-GS-HPR) (SEQ ID NO: 111) is synthesized.

This example utilized the procedure by Fujimoto et al., known as a synthetic method of long-chain DNA (Hideya Fujimoto, "the Production of Synthetic Gene", Plant Cell Technology Series 7 PCR Experiment Protocol for Plant, Shujunsha, pp.95-100 (1997)). The principle of this method is that the oligonucleotide primers on the order of 120 mer are created to have the overlaps on the order of 20 mer at the 3' end, and the deficient portion is extended utilizing the overlap regions of the oligonucleotide primers and further amplified by performing PCR using the primers at both ends. This procedure is successively repeated to synthesize long-chain DNA of interest.

A series of overlap PCR method is successively carried out according to Fig. 4. The DNA sequences of twenty various primers (Ha, H01, Hb, H02, Hc, H03, Hd, H04, He, H05, Hf, H06, Hg, H07, Hh, H08, Hi, H09, Hj, H10) shown in Fig. 4 are shown in SEQ ID NOs. 112-131, respectively. After the completion of extension reaction in Step 4 shown in Fig. 4, it is confirmed whether the full-length A01-GS-HRP gene has been synthesized.

Oligonucleotide (SEQ ID NO: 132) which is an upstream primer and oligonucleotide (SEQ ID NO: 133) which is a downstream primer, relative to the A01-GS-HRP gene, are designed and synthesized, respectively.

Those two oligonucleotides are used as a primer pair and PCR amplification is carried out using the A01-GS-HRP gene as a template to obtain, as an amplified product, DNA containing the full-length PHA synthetic enzyme gene having the BamHI restriction site upstream and the XhoI restriction site downstream (LA-PCR Kit; Takara Shuzo).

Upstream primer (SEQ ID NO: 132): 5'-AGTCGGATCC GTTTATGCGA ATCAGACTCC GCCTTCTAAG GCGCGGGGTG GAGGTTCG-3'

Downstream primer (SEQ ID NO: 133): 5'-AGGCCTCGAG AGAGTTGGAG TTCACCACCC TACA-3'

2) Gene transfer and expression of fused product

Ligation into the appropriate restriction cleavage sites (BamHI and SacI) of the pGEX-C1 plasmid for expressing the above fused protein GST-YN2-C1 is performed.

The plasmid pGEX-C1 made in Reference Example 2 and the PCR product obtained as above are digested with the restriction enzymes BamHI and XhoI.

Next, the above fragments are bound using T4 ligase.

By using this ligation solution, transformation into 40 µl of the *E. coli* solution (JM109 Competent Cell) is performed by the heat shock method. To the *E. coli* solution where transformation has been made, 750 µL of LB is further added and cultured while

being shaken at 37°C for 1 hour. Subsequently, the above cultured solution is inoculated over LB/amp. Plate and left to stand overnight at 37°C.

The confirmation of the base sequence of the insert introduced into the expression vector is carried out by determining the base sequence inserted between BamHI and XhoI sites of the restriction enzymes by the sequencing of the plasmid DNA as a template which has been prepared using Miniprep (Wizard Minipreps DNA Purification Systems, manufactured by PROMEGA), with pGEX 5' Sequencing Primer (manufactured by Amersham Pharmacia Biotech).

The resulting expression vector for the GST-A01-GS-HPR-fused protein is transformed into the BL21 (DE3) competent cell by the heat shock method. After the resulting strain for expression is pre-cultured overnight in 10 ml of LB-Amp medium, 5 ml of that cultured product is added to 1.5 L of LB-Amp medium and cultured while being stirred at 28°C for 15 hours at 150 rpm. IPTG (final concentration 1mM) is then added thereto and the culture is continued at 37°C for 12 hours.

IPTG-derived *E. coli* is harvested (8000 xg, 30 min., 4°C) and resuspended into 1/10 volume of 4°C PBS. The fungus cells is ruptured by freeze-thaw and sonication and centrifuged (8000 xg, 10 min., 4°C) to remove solid impurities. The expressed protein of

interest is confirmed to exist in the supernatant by SDS-PAGE.

3) Purification of fused protein

From the supernatant, the GST-fused protein which has been derived and expressed is purified with Glutathion Sepharose 4B beads (manufactured by Amersham Pharmacia Biotech).

It is noted that the GST-fused protein which is derived and expressed is the fused protein GST-A01-GS-HPR into which the peptide chain is inserted where the amino acid sequence of the above SEQ ID NO: 1 and the linker sequence GGGS are coupled between the C-terminal of the GST protein as a fusion partner and the N-terminal of the HRP protein.

The glutathione sepharose used is previously treated for suppressing nonspecific absorption. That is, after the glutathione sepharose is washed (8000 xg, 10 min., 4°C) three times in the equal volume of PBS, the equal volume of 4% BSA-containing PBS is added thereto and the whole is treated at 4°C for 1 hour. Following treatment, the glutathione sepharose is washed twice in the equal volume of PBS and resuspended into 1/2 volume of PBS. 400 µL of the glutathione sepharose pretreated is added to 10 mL of a cell-free extract (supernatant) and the whole is gently stirred at 4°C. By this stirring, the fused protein GST-A01-GS-HRP is absorbed to the glutathione

sepharose.

After the absorption, the glutathione sepharose is collected by centrifugation (8000 xg, 10 min., 4°C) and washed three times in 5 ml of PBS. Subsequently, 40 µl of 10 mM glutathione is added and the whole is stirred at 4°C for 1 hour to elute the fused protein which has been absorbed. The supernatant containing the fused protein is collected by centrifugation (8000 xg, 3 min., 4°C) and dialyzed against PBS to purify the GST-fused protein. Following the purification, a single band is shown by SDS-PAGE.

With PreScission protease (Amersham Pharmacia Biotech, 5U), 500 µg of each GST-fused protein is digested and the fusion partner GST portion at the N-terminal is separated by cleavage. This solution is loaded onto a glutathione sepharose column to remove protease and GST. The flow-through fraction of the glutathione sepharose column is further loaded onto the Sephadex G200 column equilibrated with PBS to obtain, as a final purified product, the peptide-fused protein A01-GS-HRP/PBS.

4) Biosensor using affinity peptide-fused HRP

The resulting A01-GS-HRP is immobilized onto the aluminum oxide-deposited platinum substrate by the following method.

The above substrate is dipped into the A01-GS-

HPR/PBS obtained as above and stirred overnight at room temperature. Subsequently, the aluminum oxide substrate is pulled out of the solution and dipped into 0.05% Tween 20/phosphate aqueous solution, followed by washing the front and back of the plate while stirring for 3 min (100 rpm). This procedure is repeated three times. This results in the HRP-immobilized substrate.

The resulting HRP-immobilized substrate as a working electrode, as well as platinum as a counter electrode and silver/ silver chloride as a reference electrode are dipped into the container filled with 30 mM of potassium iodide/phosphate aqueous solution (pH 7.4) to create the enzymatic electrode measurement system.

Next, after the potential E1=-300 mV vs. Ag/AgCl is applied to the working electrode for 40 seconds, the potential at the working electrode is changed to 0 mV vs. Ag/AgCl and maintained for 240-320 seconds, followed by stepping the potential to -300 mV vs. Ag/AgCl. As such, the potential step between 0 mV vs. Ag/AgCl and -300 mV vs. Ag/AgCl is repeated until the current behavior between the working electrode and the counter electrode is allowed to have reproducibility. Subsequently, a hydrogen peroxide solution is added such that the final concentration is brought to 10 μM, and the

value of the current of the HRP-immobilized electrode at this point is measured. Stirring is carried out for 5 seconds every addition of the hydrogen peroxide solution and after a lapse of 30-40 seconds the potential step proceeds from 0 mV to E1=-300 mV vs. Ag/AgCl. Next, the potential is stepped to E2=0 mV vs. Ag/AgCl and at this point the current response which has flowed between the working electrode and the counter electrode is monitored.

Then, current responses at final concentrations of the hydrogen peroxide solution of 50, 100, and 500 μM are monitored in the same way. As a result, increase in current of the HRP-immobilized electrode proportional to the amount of hydrogen peroxide added is observed.

The above hydrogen peroxide electrode is expected to function as a hydrogen peroxide sensor.

Example 5

Production of aluminum oxide affinity peptide-fused GroEL

An *E. coli* expression vector expressing the anodisc membrane binding peptide-GroEL fused protein which is produced by fusing the amino acid sequence showing an affinity to the anodisc membrane, Val-Tyr-Ala-Asn-Gln-Thr-Pro-Pro-Ser-Lys-Ala-Arg (SEQ ID NO: 1), to the N-terminal of the GroEL through the linker sequence GGGS is constructed as follows.

1) Production of DNA fragment encoding aluminum oxide affinity peptide-fused GroEL and preparation of primer

At first, DNA encoding the above anodisc membrane binding peptide-fused protein-linker sequence GGGS-GroEL (A01-GS-GroEL) (SEQ ID NO: 134) is synthesized.

A series of overlap PCR method is successively carried out according to Fig. 5 in the same manner as in Example 5-1). The DNA sequences of twenty in total of various primers (Ga, G01, Gb, G02, Gc, G03, Gd, G04, Ge, G05, Gf, G06, Gg, G07, Gh, G08, Gi, G09, Gj, G10, Gk, G11, Gm, G12, Gn, G13, Gp, G14, Gq, G15, Gr, G16, Gs, G17) shown in Fig. 5 are shown in SEQ ID NOs. 135-168, respectively. After the completion of extension reaction in Step 5 shown in Fig. 5, it is confirmed whether the full-length A01-GS-GroEL gene has been synthesized.

Oligonucleotide (SEQ ID NO: 132) which is an upstream primer and oligonucleotide (SEQ ID NO: 169) which is a downstream primer, relative to the A01-GS-GroEL, are designed and synthesized, respectively. Those two oligonucleotides are used as a primer pair and PCR amplification is carried out using the A01-GS-GroEL gene as a template to obtain, as an amplified product, DNA containing the full-length PHA synthetic enzyme gene having the BamHI restriction

site upstream and the XhoI restriction site downstream (LA-PCR Kit; Takara Shuzo).

Upstream primer (SEQ ID NO: 132) :

5'-AGTCGGATCC GTTTATGCGA ATCAGACTCC GCCTTCTAAG GCGCGGGGTG GAGGTTCG-3'

Downstream primer (SEQ ID NO: 169) :

5'-AGGCCTCGAG TTACATCATG CCGCCCATGC CAC-3'

2) Gene transfer and expression and purification of fused product

After the same procedure is performed as in Example 5-2) using the synthesized base sequence and the above primers, from the supernatant, the GST-fused protein which has been derived and expressed is purified with Glutathion Sepharose 4B beads (manufactured by Amersham Pharmacia Biotech).

It is noted that the GST-fused protein which is derived and expressed is the fused protein GST-A01-GS-GroEL into which the peptide chain is inserted where the amino acid sequence of the above SEQ ID NO: 1 and the linker sequence GS are coupled between the C-terminal of the GST protein as a fusion partner and the N-terminal of the GroEL protein.

The glutathione sepharose used is previously treated for suppressing nonspecific absorption. That is, after the glutathione sepharose is washed (8000 xg, 10 min., 4°C) three times in the equal volume of PBS, the equal volume of 4% BSA-containing PBS is

added thereto and the whole is treated at 4°C for 1 hour. Following the treatment, the glutathione sepharose is washed twice in the equal volume of PBS and resuspended into 1/2 volume of PBS. To 10 mL of a cell-free extract (supernatant), 400 µL of the glutathione sepharose pretreated is added and the whole is gently stirred at 4°C. By this stirring, the fused protein GST-A01-GS-GroEL is absorbed to the glutathione sepharose.

After the absorption, the glutathione sepharose is collected by centrifugation (8000 xg, 10 min., 4°C) and washed three times in 5 ml of PBS. Subsequently, 40 µl of 10 mM glutathione is added and the whole is stirred at 4°C for 1 hour to elute the fused protein which has been absorbed. The supernatant containing the fused protein is collected by centrifugation (8000 xg, 3 min., 4°C) and dialyzed against PBS to purify the GST-fused protein. Following the purification, a single band is shown by SDS-PAGE.

With PreScission protease (Amersham Pharmacia Biotech, 5U), 500 µg of each GST-fused protein is digested and the fusion partner GST portion at the N-terminal is separated by cleavage. This solution is loaded onto a glutathione sepharose column to remove protease and GST. The flow-through fraction of the glutathione sepharose column is further loaded onto

the Sephadex G200 column equilibrated with PBS to obtain, as a final purified product, the peptide-fused protein A01-GS-GroEL/PBS.

3) Enzyme activated filter using affinity peptide-fused GroEL

The resulting A01-GS-GroEL and aluminum oxide nanoholes are immobilized by the following method.

Into an aluminum oxide nanoholes-embedded syringe filter (membrane 60 μm in thickness, 10 mm in diameter, pore size 0.2 μm, trade name: Anotop 10 Syringe Filter, manufactured by Whatman), 10 ml of the A01-GS-GroEL 1 μl-PBS solution obtained as above is injected at a speed of 0.1 ml/min. Subsequently, 30 ml of 0.05% Tween 20/phosphate aqueous solution is injected at a speed of 0.5 ml/min, followed by washing. This results in the filter which optimizes the stereo structure of a protein.

On the other hand, Yeast Enolase (manufactured by Oriented Yeast Corporation) which is denatured in 4 M guanidine hydrochloride to decrease activity is added into the renaturing buffer (10 mM $Mg(CH_3COO)_2$, 20 mM KCl, 5 mM ATP, 50 mM tris-Cl, pH 7.8) such that the final concentration is brought to 15 μg/ml. The mixture is then injected into the above filter at a speed of 2 μl/min to react.

The activity of the enolase after the completion of reaction is observed to have the

ability of activity recovery, when 0.04 ml of the enolase solution is mixed with 0.96 ml of the substrate solution (50 mM tris-Cl, pH 7.8, 1mM MgCl2, 1mM 2-PGA (2-phosphoglycericacid) and increase in the absorbance at 240 nm by the production of phosphoenol pyruvate at 37°C is measured as a function of time. Comparing the enolase activity ability with GroEL composed of the subunits lacking the aluminum oxide affinity peptide, 95% of the ability of activity recovery is observed. For comparison, the ability of activity recovery of the GroEL in which the histidine tag remains added to all the subunits is examined and found to be 80% so that improvement can be confirmed.

Example 6

Aluminum oxide affinity peptide analog-fused PHA synthetic enzyme

In order to confirm that the altered amino acid sequences having the deletion, substitution, or addition of the amino acid of the aluminum oxide affinity peptide, or the amino acid sequence in combination of two of them has an affinity, *E. coli* expression vectors expressing the anodisc membrane-binding peptide-PHA synthetic enzyme fused products are constructed as follows, which are produced by fusing, to the N-terminal of the PHA synthetic enzyme through the linker sequence GGGS, the sequence having the deletion of 4 residues of the amino acid: Tyr-

Ala-Gln-Thr-Pro-Pro-Ser-Arg (SEQ ID NO: 170), the sequence having the substitution of 4 residues of the amino acid: Leu-Tyr-Ala-Gln-Gln-Thr-Pro-Pro-Ser-Arg-Ser-Arg (SEQ ID NO: 171), the sequence having the addition of 4 residues of the amino acid: Val-Tyr-Ala-Asn-Gln-Thr-Pro-Pro-Ser-Arg-Ala-Arg-Ala-Lys-Ala-Arg (SEQ ID NO: 172), and the sequence combining SEQ ID NO: 1 and SEQ ID NO: 170: Val-Tyr-Ala-Asn-Gln-Thr-Pro-Pro-Ser-Lys-Ala-Arg-Tyr-Ala-Gln-Thr-Pro-Pro-Ser-Arg (SEQ ID NO: 173), respectively, relative to the affinity peptide of the peptide-fused protein A01-GS-YN2-C1 having a high affinity as indicated in Example 3, Val-Tyr-Ala-Asn-Gln-Thr-Pro-Pro-Ser-Lys-Ala-Arg (SEQ ID NO: 1).

The DNA fragment encoding this anodisc membrane binding peptide and the linker sequence portion is created as double-stranded DNA and ligated into the appropriate restriction cleavage sites (BamHI and SacI) of the pGEX-C1 plasmid for expressing the fused protein GST-YN2-C1. In this case, the ends of the synthesized oligonucleotides of SEQ ID NO: 174 to SEQ ID NO: 181 has been phosphorylated using T4 polynucleotide kinase (manufactured by Gibco) according to the manufacturer's instruction. Subsequently, two equimolar synthesized DNAs are mixed and heated at 80°C for 5 minutes, followed by slow cooling to room

temperature, thereby forming the double-stranded DNA fragment. The formed double-stranded DNA fragment is directly used for subsequent cloning. After that, gene transfer and the expression and purification of the fused product are carried out in the same manner as in Example 3-2 to obtain 10 U/ml of purified solutions of A01M-GS-YN2-C1 in which 4 residues of the amino acid are deleted from A01-GS-YN2-C1, A01S-GS-YN2-C1 in which 4 residues of the amino acid are substituted, A01P-GS-YN2-C1 in which 4 residues of the amino acid are added, and A01W-GS-YN2-C1 in which SEQ ID NO: 1 and SEQ ID NO: 170 are combined.

In addition, the affinity evaluation for the resulting peptide-fused proteins is carried out in the same manner as in Example 3-3 and the enzymatic activity is measured. The result is shown in Table 10.

[Table 10]

Enzymatic activity of each PHA synthetic enzyme protein bound on aluminum oxide particle

PHA synthetic enzyme complex	Enzymatic activity (U)
A01-GS-YN2-C1	0.11
A01M-GS-YN2-C1	0.10
A01S-GS-YN2-C1	0.08
A01P-GS-YN2-C1	0.07
A01W-GS-YN2-C1	0.12
YN2-C1	0.01

As compared to the control enzyme protein YN2-C1, similarly to the enzyme protein A01-GS-YN2-C1 fusing, at the N-terminal, the peptide chain of the amino acid sequence having the binding ability to aluminum oxide, A01M-GS-YN2-C1, A01S-GS-YN2-C1, A01P-GS-YN2-C1, and A01W-GS-YN2-C1 are observed to have higher enzymatic activity. This showed that, of the amino acid sequences of the above aluminum oxide affinity peptide, the enzyme protein in which any of the altered amino acid sequences having the deletion, substitution, or addition of a few amino acids or the amino acid sequence in combination of two or more of them had been fused was allowed to be effectively immobilized on the surface of the substrate composed of aluminum oxide through the N-terminal where they were located.

INDUSTRIAL APPLICABILITY

In the present invention, an organic substance-immobilized structure, for example, a substrate having a surface on which a biological substance is immobilized, is provided with an aluminum oxide layer as the substrate's surface on which the substance is to be immobilized. In the organic substance to be immobilized, on the other hand, the biological substance itself is provided as a functional domain, which is coupled with a binding domain having an

ability to bind to the aluminum oxide layer. Thus, the biological substance portion provided as the functional domain can be allowed to be selectively immobilized by means of the binding ability to the aluminum oxide through the binding domain being coupled without directly contacting with the surface of the substrate. The biological substance being immobilized on the surface of the substrate through the independently-formed binding domain is free from any chemical reaction that affects the functions of the biological substance because of no influence of immobilization on the inherent functions and no reagent used for the immobilization. Therefore, in the biological substance-immobilized substrate, which can be obtained by applying the present invention, the biological substance being immobilized receives as small influence on its functions as possible, while being immobilized on the surface of the substrate effectively with high orientation.

In other words, the present invention is applicable to a technique of enhancing performance of a product by the utilization of a function of various biological substances, such as a biosensor or bioreactor in which the organic substance such as the biological substance is immobilized on the surface of the substrate, and various biological functions of the organic substance are utilized.

This application claims priority from Japanese Patent Application No. 2004-016858 filed January 26, 2004, which is hereby incorporated by reference herein.